CONTINUED VALIDATION OF SYBR GREEN-1-BASED FLUORESCENCE ASSAY FOR THE ROUTINE SCREENING OF *PLASMODIUM FALCIPARUM* SUSCEPTIBILITY TO ANTI MALARIAL` DRUGS IN GHANA.

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DECLARATION

I declare that this project work was carried out by me Mr. Dery Victor, under the supervision of Dr. Neils Ben Quashie and Dr. Francis Anto and that no previous submission for a degree in this university has been made. Related work by others which served as source of knowledge has been duly acknowledged by reference to authors.

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DEDICATION

I dedicate this work first of all to the almighty God, to my mother, my brothers Ignatius, Obed, to my sister Ruth and to my friends Christopher Adika and Ni-Iretey Christopher.

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ABREVIATIONS

ACD	Acid Citrate Dextrose
CDC	Center for disease control
DNA	Deoxyribonucleic acid
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
ICT	Immunochromatographic test
IRBC	Infected red blood cells
LDH	Lactate dehydrogenase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pLDH	Plasmodium lactate dehydrogenase
PRBC	Parasitized red blood cells
RBC	Red blood cells
RNA	Ribonucleic acid
RPMI	Roswell pack memorial institute
WHO	World health organization

ABSTRACT

This study describes the validation of a sybr green-1 based in vitro test of susceptibility of *Plasmodium falciparum* to antimalarial drugs. The assay was evaluated by determining fluorescence development at various ranges of parasitaemia of 3D7 clones incubated with sybr green in the dark. The flourescence was measured with a fluorimeter after every 1hr time interval of incubation. The effect of haemoglobin on the sybr green fluorescence was also investigated with salmon sperm DNA. The relationship between the levels of parasitemia and the sybr green fluorescence units determined for varying periods of incubation of parasite with the sybr green dye in the dark showed a well-correlated, linear relationship (r^2 =0.9962). A decreasing trend in Sybr green fluorescence with increasing haemoglobin at fixed salmon sperm DNA (1000ng/ml) was observed in this study. The quantification limit (QL) as determined by the assay was 0.5%. A z' - value of ≥ 4.7 for a test well was also determined, which was well within a quality assay acceptable range. A comparison of this fluorescence assay and a standard Giemsa stain microscopy method showed similar effective concentrations of known antimalarial drugs that resulted in a 50% reduction in the observed parasite counts (IC_{50}) after 72hrs of incubating the parasites with each drug. A positive correlation (r^2 =0.1182, P=0.1378, CI; -2.563 to 17.10), also suggest a strong antiplasmodial activity of chloroquine by both methods likewise for artesunate (r^2 =0.0098, P=0.6772, CI; -21.38 to 14.21). The sybr green-1 based assay is an easy to perform, sensitive and suited for screening of large numbers of samples. The sybr green assay proved successful for cultured 3D7 strains at 7.8% to 0.004% levels of parasitaemia and 0.2ng/µl to 0.003ng/µl of extracted DNA as well as its applicability to clinical isolates. The Sybr green assay is an easy to perform alternative, due to the substantial amount of time spent in reading slides making the microscopy method somehow a cumbersome procedure. Based on the results, it is therefore recommended that

the Sybr green assay is a reliable tool without time consumption and hazards of other screening assays and can be used for malaria drug susceptibility monitoring on the field.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

According to WHO (2012) an estimated 219 million cases of malaria with 660, 000 deaths occurred in 2010, with Africa accounting for 90% of all cases.is the most affected continent with about 90% of malaria deaths reported in 2010, mostly in children aged less than five years living in sub-Saharan Africa (WHO, 2012). Malaria epidemics kill more than 100,000 people of all ages every year (WHO 2012). According to the world malaria report (WHO, 2012), People at greatest risk are those who have been exposed to malaria infrequently and have developed little or no protective immunity. It is estimated that up to 124 million people in Africa live in areas at risk of seasonal epidemic malaria, and many more in areas outside Africa where transmission is less intense (WHO, 2012). Malaria is a major public health problem in many developing countries (Galindom *et al*., 1999). It is widespread in tropical and sub-tropical regions, including parts of the Americas, Asia, and Africa (Snow *et al*., 2005). Each year, there are approximately 350–500 million cases of malaria (W.H.O, 2005) killing 1.5 to 2.7 (Sachs *et al*., 2002) million people, the majority of whom are young children in Sub-Saharan Africa (CDC, 1993). Ninety percent of malaria-related deaths occur in Sub-Saharan Africa and malaria is commonly associated with poverty and is a major hindrance to economic development (Snow *et al.,* 2005). Malaria is one of the major causes of anaemia in children in sub-Saharan Africa, accounting for an estimated 18% of the disability adjusted life years lost because of anaemia (Murray *et al*., 1996).

Also malaria is known to account for 53.3% of all out patient cases, 41.4% of all admissions, and 41.7% of all deaths among children less than five years in Ghana (MOH,

2010). In Ghana daily and annual cases stand at 8200 and 3,000,000 respectively with 3000 deaths in 2010 (MOH, 2010).

In vitro test of susceptibilities of malaria parasites to antimalarial drugs has been used to monitor drug resistance in field isolates (Kurth *et al*., 2009; Toure *et al*., 2008). The method involves culturing of parasites in the presence of a range of drug concentrations for one life cycle or part thereof, followed by assessment of inhibition to parasite growth or multiplication (Noedl *et al*., 2003). According to Basco and colleagues, a concentration of the various drugs achieving 50% inhibition of parasite growth is usually accepted as a measure of the efficacy of the drug. Adoption of in vitro assays offers the advantage to assess the parasites' responses to drugs without interference from host factors. These factors include acquired immunity and pharmacokinetic profiles such as poor absorption, bio-transformation, concentration in certain tissues and rapid clearance (Basco, 2003). In vitro test of parasites sensitivity methods such as the [^{3}H] hypoxanthine, the morphological WHO microtest and cytometric methods have been widely used globally to conduct routine anti-malaria drug resistance monitoring but some of these methods have limitations such as radioactive waste disposal, expensive equipments as well as requiring high level of expertise (Desjardins *et al*., 1979; De Monbrison *et al*., 2003). To further enhance improvement upon these, several fluorescent dyes methods including the Sybr Green-1 dye has been considered as a cost-effective alternative to the aforementioned methods **(**Bennett *et al*., 2004).

The Sybr Green-1 assay has been validated and applied in anti-malaria drug resistance surveillance. Although fast and relatively inexpensive, growth assessment using nucleic acid stains also have inherent limitations because these stains are not specific for malaria parasite DNA (Bennett *et al*., 2004). The SYBR Green I bind to any double-stranded DNA thereby resulting in high background readings. Earlier studies have indicated that it

is an asymmetrical cyanine dye, binding to double stranded DNA, preferring G and C base pairs (Bennett *et al*., 2004). When intercalated into DNA, it is highly fluorescent, absorbing light at a wavelength between 390 and 505 nm, with a peak at 497 nm and a secondary peak near 254 nm. It emits light at 505 to 615 nm, with a peak at 520 nm (Smilkstein *et al*., 2004). Although the use of the Sybr Green method to assess the outcome of in vitro drug test has been reported the method still faces some problems and need continuous validation to bring it to perfection. Additionally the use of this method in Ghana has not been reported. It is therefore imperative for further validation of this method and its optimization for use in Ghana.

The present study aims at further validating previously validated factors which are widely believed to have an influence on the performance of the sybr green fluorescence dye.

1.2 Justification

Malaria is the most important infectious tropical parasitic disease in sub-Saharan Africa and accounts for about 20% of all infant deaths in the sub-region (WHO, 2010). Traditionally, high-throughput in vitro anti-malarial drug screening have involved the use of microscopy or radioactive substrates, such as [^{3}H] hypoxanthine, to measure parasite growth in the presence or absence of known anti-malaria drugs or test compounds. These tests have their own limitations. Microscopy which is very tedious to undertake requires the services of an expert and even that sometimes the results can be subjective. The [^{3}H] hypoxanthine uptake assay involves the handling of a radioactive substance which is hazardous. The malaria SYBR Green I-based fluorescence assay, described by Smilkstein and colleagues is an alternative method for determining in vitro parasite growth and anti-malarial drug effects (Smilkstein *et al*., 2004). Parasite growth is measured using SYBR Green I, a dye that fluorescence green when intercalated into DNA. As mature erythrocytes do not possess DNA or RNA, the dye preferentially stains the parasite DNA. The level of fluorescence is directly related to parasite growth (DNA replication and accumulation) and therefore accurate IC_{50}s can be determined for compounds that inhibit the growth of the parasite. Comparatively, the Sybr Green assay is more cost effective, quick, simple, and less hazardous, while still allowing for accurate high-throughput, automated analysis.

1.3 General Objective

To evaluate the sensitivity and factors that could affect the performance of the Sybr Green -1 dye and to validate these by determining the susceptibility of Ghanaian *P. falciparum* isolates to artesunate (AS) and chloroquine (CQ) using the Sybr Green assay and also to compare the Sybr Green assay and microscopy methods.

1.4 Specific Objectives

- To assess the correlation between parasite DNA concentration and sybr green fluorescence
- To determine the *in vitro* drug susceptibility (IC_{50}) of Ghanaian *P. falciparum* isolates to chloroquine and artesunate
- To compare microscopy and the Sybr Green-1 assay methods.

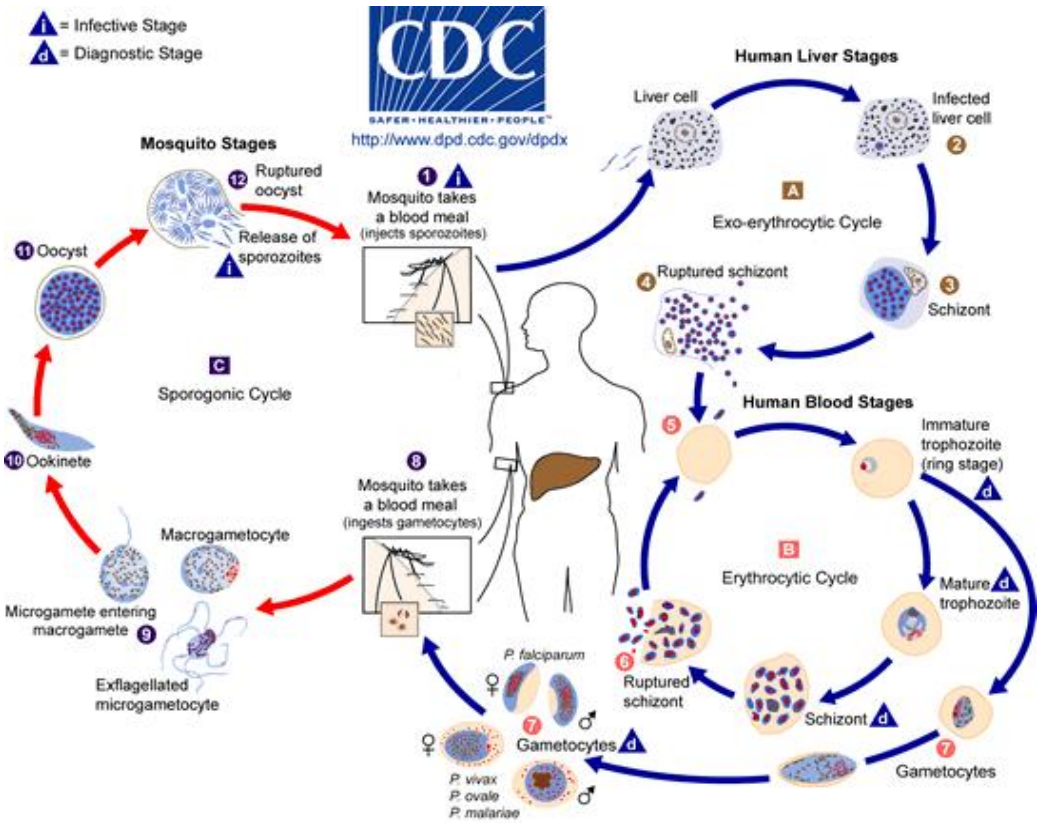
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Causes of Malaria

Malaria is caused by a protozoan parasite of the genus *Plasmodium*. The plasmodia parasites are transmitted from one person to another by the female Anopheles mosquito (www.cdc.gov/malaria/about/biology/mosquitoes). *Plasmodium* develops in the gut of the female *Anopheles* mosquito and passed on in the saliva of the mosquito to an uninfected person each time it takes a blood meal (Capanna, 2006). Malaria transmission can also occur through blood transfusion (Kitchen, 2006; Diop *et al.,* 2009; Mungai, *et al.,* 2001), organ transplant and from mother to child in congenital malaria (Lee *et al*., 1994; Menendez, 1995). Blood entering the victim's liver then carries the parasites to the liver where they invade the parenchyma cells of the liver, here they usually develop into exo-erythrocytic schizonts and multiply in the liver cells over the course of one to two weeks, to form 30,000 to 40,000 merozoites (http://www.pdp.cdc.gov/dpdx.). Exoerythrocytic schizont is sometimes referred to as the secondary tissue schizont and is absent in most important human malaria cases. After 9-16 days, they return to the blood and penetrate the red cells constituting the pre-erythrocytic schizonts (primary tissue schizont), where they multiply again, and form merozoites, progressively breaking down the red cells and this induces bouts of fever in the infected individual. The *plasmodium* parasite can invade the brain where it causes cerebral malaria in this case; the infected red cells obstruct the blood vessels in the brain.

The five species of the *Plasmodium* parasites that are known to infect humans include *P. falciparum, P. malariae, P. vivax, P. Ovale* and *P. knowlesi*. The most serious form of the disease is caused by *P. falciparum*, while P. *vivax*, *P. ovale* and *P. malariae* cause milder disease in humans. A fifth species, *P.knowlesi*, (Singh *et al*., 2004 and Collins *et al*., 2009)



Legend

Δi =Infective stage  = Liver stage (merozoites) ♀♂= Male and female gametes

Δd=Diagnostic stage =Blood stage  =Mosquito stage (release of sporozoites)

While the four major species of *Plasmodium* differ in some ways from each other, they all share the same complex life cycle involving the insect (mosquito) vector and the human host.

2.2 Global Burden of Malaria

Malaria epidemics kill more than 100,000 people of all ages every year (WHO 2012). People at greatest risk are those who have been exposed to malaria infrequently and have developed little or no protective immunity. It is estimated that up to 124 million people in Africa live in areas at risk of seasonal epidemic malaria, and many more in areas outside Africa where transmission is less intense (WHO, 2012). Malaria is a major public health problem in many developing countries (Mohga *et al*., 2012). It is widespread in tropical and sub-tropical regions, including parts of the Americas, Asia, and Africa (Snow *et al*., 2005). Each year, there are approximately 350–500 million cases of malaria (W.H.O, 2005) killing 1.5 to 2.7 (Sachs *et al*., 2002) million people, the majority of whom are young children in Sub-Saharan Africa (CDC, 1993). Ninety percent of malaria-related deaths occur in Sub-Saharan Africa and malaria is commonly associated with poverty and is a major hindrance to economic development (Snow *et al.*, 2005). Malaria is one of the major causes of anaemia in children in sub-Saharan Africa, accounting for an estimated 18% of the disability adjusted life years lost because of anaemia (Murray *et al*., 1996). The number of deaths attributable to malaria-associated anaemia is estimated at 190,000 to 974,000 a year. In high-intensity transmission areas, the highest burden of malaria and malaria-associated anaemia is in infants (Schellenberg *et al*., 1999; Murphy *et al*., 2001).

According to World Malaria Report, the percentage of malaria infection in Africa is estimated as 70% (W.H.O, 2002). In the Kassena-Nankana District of northern Ghana, malaria infection in children 2-12 months old according to Binka and colleagues is 8.6% in June 2000 and 52.8% in November 2000 (Binka *et al*., 2005).

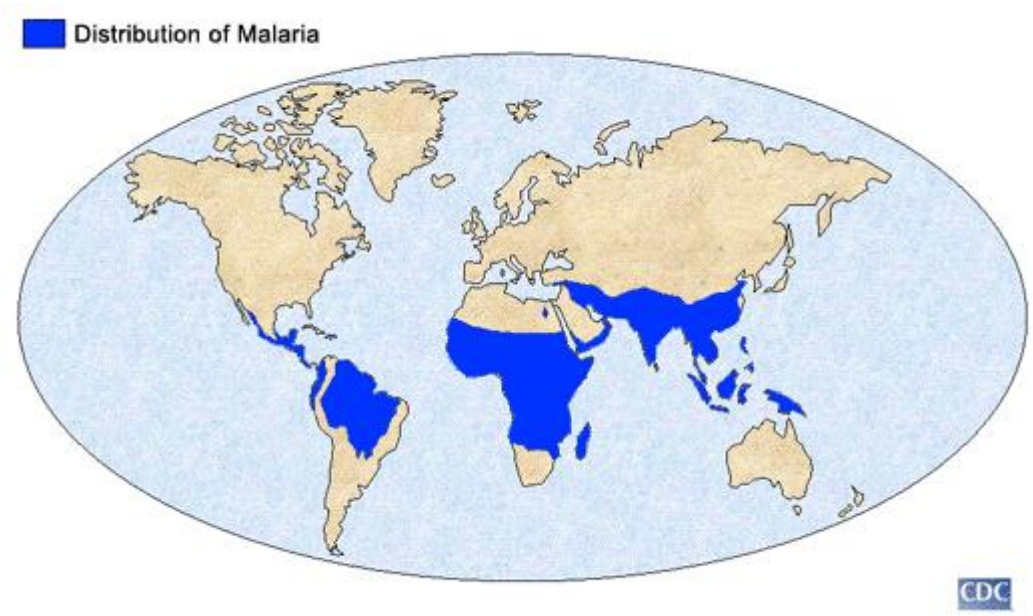


Fig 2: Worldwide Geographical Distribution of Malaria

Source: http://www.cdc.gov/malaria/images/graphs/geodistribution.gif.

Legend: = Malaria endemic areas = Malaria free areas

2.3 Economic Burden

The malaria transmission season generally coincides with the planting and/or harvesting season and brief periods of illness exact a high cost on the world's poorest regions (Chitsulo et *al*., 1994; CDC, 2005). An estimated decrease in economic growth due to malaria in disease endemic countries is greater than one percentage (1%) point per year (Malaney *et al.,* 2002). Malaria remains inextricably linked with poverty. The highest malaria mortality rates are being seen in countries that have the highest rates of extreme poverty (proportion of population living on less than US$ 1.25 per day (WHO, 2012).

2.4 Treatment of Malaria

In the world malaria report, treatment of malaria depends on the species of the infecting parasite (W.H.O, 2006). Infection with *P. vivax, P. ovale* or *P. malariae* can often be treated on an outpatient basis while infection with *P. falciparum* is associated with severe malaria and can be fatal. The density of the parasite in the bloodstream and other relevant factors for treatment include the parasite's drug resistance status and the country where the parasite was contracted (CDC, 2006). Treatment also depends on any accompanying illnesses and drug allergies. The center for disease control (CDC) suggestions are that treatment should begin within 24 hours after symptoms appear, particularly with a *P. falciparum* infection because of its rapid progression towards severe malaria (CDC, 2006) .The drug(s) administered depends on the identified parasite species and drug resistance in the region where the parasite was acquired. Travel history is especially crucial in the identification and drug resistance process. Anti-malaria drugs can be given orally, intravenously, or as a suppository determined by the severity of the infection. Most drugs are given when the parasite has a high density in the bloodstream. Medication can be administered for patients who have *P. falciparum* for effective treatment by giving

continuous intra-venous (IV) infusion as the best alternative because some people might not take the drug or vomit upon taking it.

The most effective new drugs are the artemisinins. According to the world health organization report the following artemisinin-based combination therapies (ACTs) are been recommended:

• Artemether-lumefantrine, (AS+AL)

• Artesunate + amodiaquine, (AS+AQ)

• Artesunate + mefloquine, (AS+AM)

• Artesunate + sulfadoxine–pyrimethamine (AS+SP) (W.H.O, 2006).

The artemisinin compounds are active against all four species of malaria parasites that infect humans and are generally well tolerated. They are currently the most effective agents against multi-resistant malaria strains. Ironically, they are derived from compounds from the most ancient treatment such as qinghao tree (annual Chinese wormwood, *Artemesia annua*). Artemisinins clear parasites more rapidly than quinine during the first 24 hours of treatment. The treatment of choice for uncomplicated *falciparum* malaria is the artemisinin combination therapy (ACTs).

The choice of ACT in a country or region is based on the level of resistance of the partner medicine in the combination: in areas of multi drug resistance (South-East Asia), artesunate + mefloquine or artemether-lumefantrine is recommended and in Africa, artemether-lumefantrine, artesunate + amodiaquine; artesunate + sulfadoxine-pyrimethamine (Nosten, 2007). In addition to the W.H.O report on malaria treatment guidelines, the artemisinin derivative components of the combination must be given for at least 3 days for an optimum effect (W.H.O, 2006). Artemether-lumefantrine should be

used with a 6-dose regimen. Amodiaquine + sulfadoxine-pyrimethamine may be considered as an interim option in situations where ACTs cannot be made available. However, these drugs are very expensive and are not yet widely available. Resistance to anti-malarials has been documented for *P. falciparum, P. vivax* and *P. malariae* (White, 2004). In *P. falciparum*, resistance has been observed to most of the anti-malarials (amodiaquine, chloroquine, mefloquine, quinine, and sulfadoxine–pyrimethamine) except for artemisinin and its derivatives. The geographical distributions and rates of spread have varied considerably. *P. vivax* has developed resistance rapidly to sulfadoxine–pyrimethamine in many areas. *P. vivax* remains sensitive to chloroquine in South-East Asia, the Indian sub-continent, the Korean peninsula, the Middle East, northeast Africa, and most of South and Central America.

2.4.1 Treatment of severe falciparum malaria.

The world malaria report (WHO, 2012), recommended treatment guideline of severe *falciparum* malaria preferred regime alternatives as follows, for adults, artesunate IV or IM:

■ Artemether or quinine is an acceptable alternative if parenteral artesunate is not available.

For children, artesunate IV or IM:

■ Artemether or quinine is an acceptable alternative if parenteral artesunate is not available.

Give parenteral antimalarials in the treatment of severe malaria for a minimum of 24 h, once started (irrespective of the patient's ability to tolerate oral medication earlier) and, thereafter, complete treatment by giving a complete course of:

- an ACT;
- Artesunate plus clindamycin or doxycycline;
- Quinine plus clindamycin or doxycycline.

If complete treatment of severe malaria is not possible, patients should be given pre-referral treatment. Intravenous (IV) artesunate should be used in preference to quinine for the treatment of severe *P. falciparum* malaria in adults. t. The further recommended the following options for pre-referral treatment: rectal artesunate, quinine IM, artesunate IM, Artemether IM (WHO, 2012).

2.4.2 Treatment of uncomplicated P. falciparum

Artemisinin-based combination therapies should be used in preference to sulfadoxine pyrimethamine (SP) plus amodiaquine (AQ) for the treatment of uncomplicated *P. falciparum* malaria. Artemisinin-based combination therapies (ACTs) should include at least 3 days of treatment with an artemisinin derivative. Dihydroartemisinin plus piperaquine (DHA+PPQ) is an option for the first-line treatment of uncomplicated *P. falciparum* malaria worldwide. Addition of a single dose primaquine (0.75 mg/kg) to ACT treatment for uncomplicated falciparum malaria as an antigametocyte medicine, particularly as a component of pre-elimination or an elimination programme (WHO, 2012).

2.5 Prevention and Control of Malaria

The control of malaria and anaemia depends largely on passive case detection and appropriate treatment. However, many children who have malaria, anaemia, or both remain asymptomatic, and chronic asymptomatic parasitaemia increases the risk of severe anaemia (Schellenberg *et al.,* 2003). Chemo-prophylaxis during the first year of life significantly reduced the incidence of malaria and anaemia in a study in Tanzania (Alonso *et al*., 1997).

Malaria transmission can be reduced by preventing mosquito bites with mosquito nets and insect repellents, or by mosquito control measures such as spraying insecticides inside houses and draining standing water where mosquitoes lay their eggs and research into development of malaria vaccines with limited success and more exotic controls, such as genetic manipulation of mosquitoes to make them resistant to the parasite have also been considered (Kondoh *et al.,* 2007).

Although some are under development, no vaccine is currently available for malaria that provides a high level of protection; preventive drugs must be taken continuously to reduce the risk of infection. These prophylactic drug treatments are often too expensive for most people living in disease endemic areas. Most adults from disease endemic areas have a degree of long-term infection, which tends to recur, and also possess partial immunity (resistance); the resistance reduces with time, and such adults may become susceptible to severe malaria if they have spent a significant amount of time in non-endemic areas. They are strongly recommended to take full precautions if they return to an endemic area. Malaria infections are treated through the use of anti- malarial drugs, such as quinine or artemisinin derivatives. However, parasites have evolved to be resistant to many of these drugs. Therefore, in some areas of the world, only a few drugs remain as effective treatments for malaria (W.H.O, 2006). In an effort to come out with an effective treatment

regimen of malaria, a randomized comparative study of chloroquine, amodiaquine and sulphadoxine-pyrimethanine for treatment of complicated malaria has been carried out in one of the selected district (Kassena-Nankana District) of northern Ghana where infection is high.

Considering the ever-increasing infection rate of malaria across the globe, more particularly Africa, there is the need for the establishment of various control and preventive models. The use of treated bed-nets and curtains (Curtis, 1991; Legeler, 2004), cluster randomized placebo control trial intermittent preventive treatment with sulphadoxine-pyrimethamine targeting the transmission season are preventive and control measures (Dicko *et al.*, 2004). Other models such as randomized comparative study of chloroquine,amodiaquine and sulphadoxine-pyrimethamine for treatment of complicated malaria (Oduro *et al*., 2005) and currently the trial of whole parasite vaccine (Biotechnology company sanaria) can be said to include preventive and control strategies aimed at the eradication of malaria.

Despite the tremendous effort at malaria eradication, some control measures or projects have failed for various reasons among some of which are deteriorating public health services, the development of resistance to available drugs by the parasites, development of resistance to insecticides and by the vector (Mueller *et al*., 1990) and human practices that enhances transmission have also been reported (Hemingway *et al.*, 2002).

2.6 Methods of Diagnosing Malaria

2.6.1 Conventional Light Microscopy

Giemsa stained thick blood film (G-TBF) is usually used to screen for the presence of parasites (Giemsa, 1904). A thin blood film helps to determine the species. Giemsa stain is specific for the phosphate groups of DNA and attaches itself to regions of DNA where there are high amounts of adenine-thymine bonding. Giemsa stain is a classical blood film stain for peripheral blood smears and bone marrow specimens (Giemsa, 1904). However, during the staining of a G-TBF 60–80% of parasites may be lost (Dowling & Shute, 1966). The detection limit of 5– 20 parasites/ml for thick blood film is thus only ten times better than for thin blood film even though the thick blood film requires a 50-times greater volume (Bruce-Chwatt, 1984). Light microscopy can routinely detect parasitaemia levels as low as 40 parasites/µl and experienced microscopists can detect as low as 5–10 parasites/µl of blood (W.H.0, 2000). Conventional microscopy allows the identification of all four *Plasmodium* species, the quantification of parasites and determination of other prognostic factors. In countries with imported malaria, expertise and continuous training in examining blood films is required (Payne, 1988). G-TBF examinations of 100 fields may miss infections of up to 20% (Caraballo *et al.,* 1996; Craig & Sharp, 1997; Kodisinghe *et al.,* 1997; Di Perri *et al.,* 1997). At least 200 fields of G-TBF should be screened before reporting a negative result (Bain *et al.,* 1997).

2.6.2 The Role of Light Microscopy in Malaria Control

Reviewing on alternatives to conventional microscopy on malaria diagnosis the W.H.O 2005 report indicates that the first suspicion of malaria is almost always based on clinical criteria and in many situations, symptom-based diagnosis is the sole basis for treatment in areas where malaria is endemic and this usually results in all patients with fever and no other apparent causes of disease being treated for malaria. Although this approach can identify most patients that need malaria treatment, it is also likely to misclassify many who do not, resulting in patients with other diseases receiving malaria treatment. While this might have been acceptable in the past when malaria was treatable with affordable and relatively safe drugs, it is not recommended in areas where microscopy is available (W.H.O, 2005). In the report it has further indicated that a diagnosis based on clinical symptoms alone has very low specificity. As a result, malaria can be over-diagnosed considerably, while other diseases are overlooked and not treated in a timely manner. This contributes to the misuse of anti-malarial drugs, increased costs to the health services and patient dissatisfaction. Indaratna and Plasai have been cited to suggest that good clinical practice dictates that a laboratory should confirm the presence of parasites in most epidemiological situations (Indaratna *et al*., 2005). Laboratory diagnosis to confirm the presence of parasites is particularly desirable in all suspected cases of treatment failures and severe disease, as well as for diagnosing uncomplicated malaria during low transmission seasons (WHO, 2000).

Laboratory diagnosis by microscopy examination of stained blood smears continues to be the method of choice (the gold standard) for confirming a clinical diagnosis of malaria and epidemiological studies (WHO 2000a, 2004a) and Parasite diagnosis is essential during clinical and field trials of anti-malarial drugs and vaccines.

2.6.3 Fluorescent Microscopy

Mature erythrocytes do not normally contain DNA and RNA, while malaria parasites do. This has led to the use of fluorescent dyes, mainly acridine orange (AO), to detect parasites. The differential staining characteristic of benzothiocarboxypurine, which does not stain viable leucocytes but stains intra-erythrocytic parasites, was applied in this direction (Makler *et al.*, 1991). A review in the Gambia showed good sensitivities, although some expertise in reading the slides was required (Hunt-Cooke *et al.*, 1993). The process has been further enhanced by the development of a fluorescent microscope fitted with an interference filter and used AO to stain thin blood films (AO-tibf) (Kawamoto 1991). Some authors found fluorescent stains comparable to G, with a slightly lower sensitivity (Wongsrichanalai *et al.*, 1992b), others reported a much higher sensitivity (Hind *et al.*, 1994). Studies highlighted problems using the equipment developed by Kawamoto, with 85% agreement between AO-tibf and G-TBF; or concluded that Giemsa stained films could not be dispensed with (Metzger & Nkeyi, 1995). Some other studies compared AO stained thin and thick blood films with various methods (G-TBF, QBC®) and found sensitivities around 90%, which decreased to 50% at lower parasitaemia levels (Gay *et al.*, 1996; Craig *et al.*, 1997). AO stained slides require special equipment, although the staining itself is cheap and expertise in reading slides is necessary (Warhurst and Williams 1996; Hunt-Cooke *et al.*, 1993).

Series of fluorescent dyes were tested for their ability to provide nucleic acid-specific fluorescence staining of malaria parasites within a red cell and that could easily be visualized using one of three standard filter sets: Ex. 340–380, BA 435–485 ("Fluorescein"); Ex 450–490/Em 520 ("DAPI"); and Ex 546/Em 590 ("rhodamine") (Rebecca *et al*., 2007) the dyes produced intense fluorescence that was easily visible. The optimal concentration of these dyes under the conditions employed was found to be a

1:500 dilution, with the exception of Sybr Green- 1, which produced strong fluorescence at a 1:10,000 dilution. Dyes that demonstrated good nucleic acid-specific fluorescence were tested in combination with Giemsa stain. No fluorescence was observed with any of the dyes when simultaneous staining of Giemsa and fluorescent dye was performed, or when fluorescence staining preceded Giemsa staining. However, dual staining was observed with Sybr Green -1, YOYO-1 and Ethd-2 dyes when the blood films were first stained with Giemsa, followed by staining with the fluorescent dye. Four factors were found to be important for optimal dual staining: these were, the blood films had to be stained with Giemsa first, followed by the fluorescent dye; Secondly particulates within the Giemsa stain had to be removed from solution by centrifugation at 10,000 × g for 2 min; A dilution of 1:25 of the Accustain® Giemsa Stain, (SIGMA-Aldrich) was optimal for staining of culture films and however most importantly, the culture film had to be hydrated prior to fluorescent staining as no fluorescent staining was observed if the cell film was allowed to dry prior to addition of Sybr Green -1.

Sequential Giemsa plus Sybr Green- 1 staining of pRBCs as described herein provides a method of rapid screening and detection of the pathogen because the bright fluorescence of nuclear staining with Sybr Green -1 is well contrasted with the low RBC auto fluorescence and the dark background. The combination of Sybr Green -1 for enhanced detection plus Giemsa for traditional identification and speciation appears to offer a superior diagnostic test.

2.6.4 Fluorescent Microscopy after Centrifugation (QBC®)

Fluorescent microscopy after centrifugation *(QBC®)* method involves an AO-coated capillary filled with 50–100 ml blood which is then centrifuged and examined under a fluorescent microscope (Levine, Wardlaw & Patton 1989). First reports of an 8–1000-times increased sensitivity as compared with G-TBF have been disputed (Wongsrichanalai *et al.,* 1992c) and sensitivity greater than 90% when compared to microscopy (Wongsrichanalai *et al.,* 1992a; Benito *et al.,* 1994; Lowe *et al.,* 1996; Craig *et al.,* 1997). However, some authors have reported sensitivities of only 84% (Caraballo *et al.,* 1996) or as low as 55% (Peterson & Marbiah, 1994). Some reports have used QBC® as the new standard (Long *et al.,* 1995) but other authors regarded the technique as complicated and demanding a lot of training (Craig *et al.,* 1997). Although speed and time saving with the QBC® is acknowledged by most investigators (Wongsrichanalai *et al.,* 1992a; Benito *et al.,* 1994; Gay *et al.,* 1996; Lowe *et al.,* 1996; Craig *et al.,* 1997), three main disadvantages have been highlighted; (i) high cost of capillaries and equipment, (ii) difficulty in species identification and quantification and (iii) technical problems, broken capillaries and the impossibility of storing capillaries for later reference. These problems make it unattractive for laboratories that process few specimens (Wongsrichanalai *et al.,* 1992c; Warhurst *et al.,* 1996).

2.7 Biochemical Methods

Considering the importance of diagnosing malaria other alternatives to the conventional microscopy method include the biochemical methods. Research conducted by Knobloch and Henk likewise by Jelinek and co-workers shows that lactate dehydrogenase from malaria parasites (pLDH) metabolizes 3-acetyl pyridine NAD (APAD) much faster than human erythrocyte LDH(Knobloch & Henk 1995; Jelinek *et al* 1996). However, two field studies produced poor results, with sensitivities of 76% and 58%, low specificity and low correlation between pLDH values and parasitaemias. Further studies shows that antibodies to asexual malaria parasites appear some days after invasion of the erythrocytes and may persist for months (Makler, 1998), the technique has therefore been developed into an antigen detection dipstick assay, OptiMAL® (for the detection of *P. falciparum and P. vivax* and a new OptiMAL 2 is under development that would allow detection of all four species. It may also be useful in the diagnosis of tropical splenomegaly syndrome or for retrospective confirmation of empirically treated non-immunes.

2.7.1 Antigen Detection Assays in Dipstick Format

The biochemical methods also serve as an alternative to conventional microscopy method. The histidine-rich protein 2 (HRP-2) in *P. falciparum* infections has led to a simple, rapid dipstick assay, the Parasight®-F (Shiff *et al*., 1994) and to another equally simple test, the ICT Malaria Pf® (Garcia *et al*., 1996) . These only detect infections with *P. falciparum.* However, manufacturers are developing methods to include other species, in particular *P. vivax*. However the rapid dipstick assay has its short falls as after a successful treatment, a high proportion of patients continue to have HRP-2 antigenaemia despite a microscopical and clinical cure. This continues for up to 7–14 days (WHO, 1996; Kodisinghe *et al.,* 1997; Mharakurwa & Shiff, 1997) and precludes their use in immediate follow up to confirm a cure (WHO, 1996; Mharakurwa, Manyame. & Shiff, 1997). Using the positive

Parasight®-F test, reasons for a false positive could be due to the presence of rheumatoid factor (RF) as many as 60% of patients who did not have malaria but were RF-positive, had a false positive Parasight®-F test (Laferi *et al.*,1997). Other newer immunochromatographic ICT Malaria Pf® test seems to perform similarly well. In six studies comprising 1346 assays, sensitivity ranged 80–100% (Pieroni *et al.*, 1998). Although this assay uses a different antibody it seems reasonable to assume that many of the problems with Parasight ®-F will apply equally. An exception might be the false positive rate with RF, which seems to be lower for the ICT Malarial Pf® (Bartolini, Saatinelli & Benucci, 1998; Grobusch *et al.*, 1999). Importantly, in contrast to the Parasight®-F test, some reagents for the ICT-test must be stored at 2–8°C (Pieroni *et al.*, 1998).

2.7.2 PLDH Assay: OptiMAL®

The *PLDH assay: OptiMAL®* as one of the biochemical methods characteristics of the parasite pLDH, already used to quantify malaria parasites, have been combined with monoclonal antibodies against species specific pLDH. The test is only positive when viable parasites are present. The dipstick contains a *P. falciparum* specific and a pan-specific antibody against all four species. However, the specific antibody, as indicated by the manufacturers, has so far only been shown to detect *P. vivax* infections reliably (Makler *et al.*, 1998; Palmer *et al.*, 1998). A field trial of the method in Honduras shows an overall sensitivity of 93% which decreased at parasitaemia levels below 100/ml (Palmer *et al.*, 1998). Preliminary reports by John and co-workers suggested that the sensitivity and specificity is similar to the HRP-2 assays, with similar limitations at lower parasitaemia levels (John *et al.*, 1998). The presence of RF also causes false positives, although on a much smaller scale and a definite advantage is the potential usefulness in the

follow-up of treated patients to confirm a cure, as the test only detects viable parasites (Grobusch *et al.,* 1999).

2.8 Molecular Techniques.

Molecular techniques are known for their superior sensitivity and may ultimately prove to be the gold standard for malaria diagnosis. The use of the SSUrRNA gene as a DNA target is another diagnostic advancement, because it allows amplification of both conserved (within the genus *Plasmodium*) and variable regions, thus enabling species differentiation. The advancement of polymerase chain reaction (PCR), has replaced the initial hybridization methods which were too cumbersome to be practical, with relatively poor sensitivities (Weiss, 1995). Most PCR assays were developed to detect *P. falciparum*, although many assays detect several or all four species. Assay performance, processing protocols and extraction procedures are still being refined and simple detection systems, like chemoluminescent techniques or simple colorimetric assays, avoiding the use of isotopes or ethidium bromide are being developed (Arai *et al.,* 1994; Laserson *et al.,* 1994; Oliveira *et al.,* 1996; Seesod *et al.,* 1997).

PCR assays can detect fewer parasites than well-performed G-TBF (Snounou *et al.,* 1993; Seesod *et al.,* 1997). This was convincingly demonstrated by reanalyzed PCR positive/G-TBF negative samples ('false' positives) by either extensive microscopy or different PCR assays (Laoboonchai *et al*., 2001). This frequently confirmed that 'false' positives were in fact true positives. Another advantage of PCR is the significantly improved species identification in mixed infections when compared with microscopy (Snounou *et al.,* 1993; Tirasophon *et al.,* 1994; Oliveira *et al.,* 1996). Although PCR can detect cases with lower parasitaemias, PCR assays may miss some cases, even with high parasite numbers (Kain *et al.,* 1993; Long *et al.,* 1995). Problems include PCR inhibitors, DNA degradation or genotypic variants (Barker, 1994; Tirasophon *et al.,* 1994). A theoretical concern that PCR

does not distinguish between viable and non-viable parasites does not seem to have been reported in any field study as a problem. The time needed to perform most PCR assays prohibits their use in routine diagnostic situations. However, in suspicious but microscopically negative cases or if species identification is of importance, PCR may be useful. Automation may make it attractive for screening large numbers.

2.9 Flow Cytometry

Flow cytometry offers the possibility of rapid enumeration of parasitaemia (Bianco *et al*., 1986). It relies on staining the parasite DNA to distinguish between infected and non-infected red blood cell (RBC) populations (Whaun *et al*., 1983). Reviewing the methods Hoechst 33258 fluorescent dye and flowcytometry were used to analyze 700 samples from Thailand and compared with Giemsa stained blood film (G-TBF) (200 fields). Both techniques detected 44 positive samples, while five remained unconfirmed by G-TBF (Van-Vianen *et al.,* 1993). Although flow cytometry offers automated counts for parasitaemias, this is offset by a rather low sensitivity of 10-times inferior to GTBF owing to 'background noise' caused by stained RNA in reticulocytes (Janse & Van Vianen 1994).

2.10 Automated Blood Cell Analyzer

An alternative to conventional microscopy in malaria diagnosis has included the automated blood cell analyzer. This new approach may be for the detection of additional cases, in which clinical suspicion did not lead to a specific request for a malaria test (Mendelow *et al*., 1999). The usefulness of these instruments for malaria diagnosis (Coulter STKR, VCS, Beckman & Coulter, Luton, UK; Technicon H1, Bayer Diagnostics, Newbury, UK) have shown that the commonest change was an increase above 3% of large unstained cells (LUC) and thrombocytopenia. Unfortunately both are unspecific and were only present in 60– 70% of malaria cases and can obviously occur in many other diseases (Fialon *et al.* 1991; Giacomini *et al.* 1991; Butthep & Bunyaratvej, 1993). The Cell-Dyn (CD) 3500 (Abbott-Diagnostics, Maidenhead, UK) seems to detect malaria pigment in monocytes and neutrophils (it might also detect pigment in schizonts and gametocytes), during routine automated blood counts (Mendelow *et al.,* 1999). Since the instrument only shows the presence of abnormal monocyte/neutrophil populations, microscopy is necessary to determine species and parasitaemia. The sensitivity will depend on the amount of pigment present in the circulation and early infections, when schizont numbers and malaria pigment is less abundant, sensitivity might be missed (Makler *et al*., 1998). A newer model, the CD 4000, has an additional channel in which RBC are lysed and then stained with propidium iodide (Kim *et al.*, 1998). Liberated malaria parasites would also stain and this might increase the sensitivity. Furthermore, as malaria pigment in white blood cells seems to be a sensitive indicator of prognosis (Silamut *et al.*, 1993; Metzger *et al.*, 1995) the instrument may offer a new way to assess disease severity.

CHAPTER THREE

3.0 METHODS

3.1 In-vitro Cultivation of Laboratory Strains of *P. falciparum*

3.1.1 Microscopic Blood Examination

A 10% fresh Giemsa stain dilution was prepared once daily using Giemsa stock at pH 7.2. Staining of only thick smear slides was done for 10-15minutes. The Giemsa –stained thick and thin blood films were examined at 1000x magnification to identify the parasites and to determine the parasite density. Parasite density was expressed as the number of asexual parasite per µl of blood calculated as follows: Parasite density (per µl) = (number of parasites counted against 100WBC x number of leucocytes (WBC) counted divided by 100)

$$\text{Parasites per µl of blood} = \frac{\text{parasites counted against 100WBC} \times \text{WBC counted}}{100}$$

3.1.2 Preparation of Complete Parasite Culture Media (1L)

Complete parasite culture media was prepared by dissolving 10.43g of powdered RPMI 1640 (RPMI 1640 with L-glutamine) with 850ml cell culture water in 1L volumetric flask. All the chemicals were ordered from sigma Aldrich Inc except Albumax™ I which was ordered from Gibco; Invitrogen, USA. 7.15g of HEPES (final concentration = 0.03 mM), 2g of Dextrose (final concentration = 0.011 mM) were dissolved in the RPMI 1640 solution. 2ml of hypoxanthine (final concentration =0.18mM) and 6.4ml of 7. 5% sodium bicarbonate (final concentration = 0.89mM) were added to the RPMI 1640 solution. Volume of the solution was then increased to the 1L mark with the cell culture water. The Solution was stirred with a magnetic stirrer until reagents were completely dissolved. P^H of medium was checked with a calibrated pH meter of Specifications = 7.0 ± 0.3. In a

sterile Laminar flow hood, medium was filtered sterile by filtration using 0.22µM filters. Ten milliliters (10ml) of 10% sterile Albumax™ I (lipid enriched bovine serum albumin) of final concentration 1.5mM was added to 200ml of the medium in the Laminar flow hood as the final step and medium stored at 4 °C until use.

3.1.3 Processing of Red Blood Cells for Parasite Culture

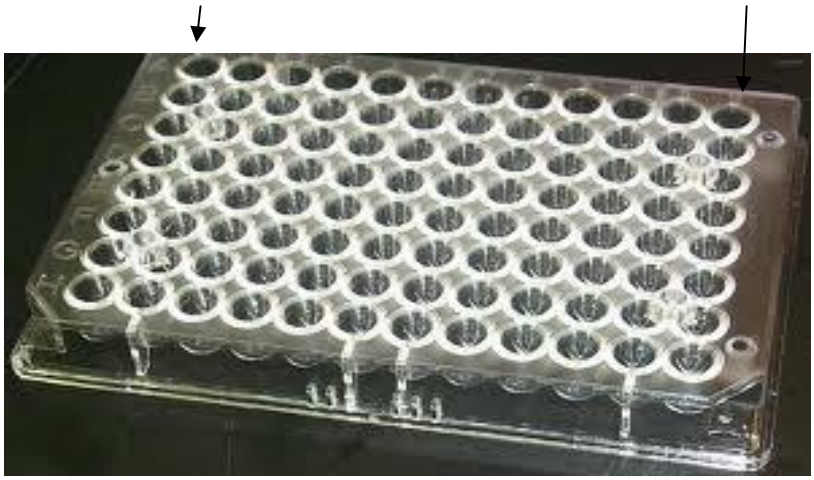
Fresh human whole blood used for the parasite cultures was donated by colleagues in the laboratory. The blood was washed to remove the plasma, buffy coat and anticoagulant by centrifugation at 630×g, 10 min, followed by removal of the supernatant and re-suspension of the pellet in an equal volume of incomplete RPMI 1640. The washing was repeated twice more, and then the erythrocytes were finally re-suspended in an equal volume of incomplete RPMI 1640 (approx. 50% packed cell volume (PCV).

3.1.4 Culturing of 3D7 P. falciparum

Human erythrocytes infected with *P. falciparum* were maintained in continuous culture using standard method (Trager & Jensen, 1976). The culture consisted of 2% suspension of human erythrocytes in complete RPMI 1640 medium. The culture was kept at 37°C under a gas mixture of 2% O_2, 5.5% CO_2, and 92.5% N_2. Determination of parasitaemia and changing of the medium was done daily. The culture was kept below 5% parasitaemia at all times.

3.1.5. Determination of Assay Quality (Z').

The statistical test published by (Zhang *et al*., 1999) was used to determine assay quality (or Z′). It is calculated as follows: $Z' = 1 - [(3\sigma_{(+)} + 3\sigma_{(-)})/ \mu_{(+)} - \mu_{(-)}]$, where $\mu_{(+)}$ and $\sigma_{(+)}$ are the mean and standard deviation of the infected erythrocytes respectively; $\mu_{(-)}$ and $\sigma_{(-)}$ are the mean and standard deviation of the noninfected erythrocytes (positive control), respectively, and the denominator value is the absolute value of the difference in the infected erythrocytes and positive control means. Assays that display a Z′ value of ≥ 0.5 are generally acceptable for high-throughput screening (Zhang *et al*., 1999).



lysis buffer/Sybr green mix to each well to make up 200µl in the wells. To setup a control, 100µl of uninfected blood (blood without DNA) as positive control was added to 100µl of the lysis buffer and fluorescence mix in row D and 100µl of the lysis buffer and fluorescence mix were added to 100µl of PBS into wells of row E to serve as the background readings. Plates were read after incubating in the dark for five minutes after the addition of the Sybr green and lysis buffer mix. After the first reading, plates were then further incubated in the dark and read at1hour interval of incubation. Fluorescence reading was measured at 485 nm (excitation) and 528 nm (emission) using the BMG labtech fluostar optima analyzer. Fluorescence readings were plotted against parasitaemia to determine the correlation. In another experiment the plates were frozen for at least one hour before observing the fluorescence readings.

3.2.2 Effect of whole Blood on sybr Green Fluorescence

Uninfected blood was washed in RPMI1640 media to remove most of the white blood cells. The washed blood haematocrit at 50% was reduced to 5% by taking 0.5ml of the washed blood in to 4.5ml of culture media. Serial dilution of the blood at 5% Haematocrit was performed to obtain haematocrit from 5% to 0.1%. Hundred microliters (100µl) of each haematocrit were dispensed into wells of column 2 through 11 of row A, B, C of the 96-well microtest plate in triplicate. Hundred microliters of 1×PBS and sybr green mixture with salmon sperm DNA at a fixed concentration of 1000ng/ml was added to the blood in the wells. To setup a control, 100µl of blood without salmon sperm DNA as the positive control was added to 100µl of the 1×PBS and sybr green mixture in wells 2 through 11 of row D and 100µl of the lysis buffer and sybr green mixture were added to 100µl of PBS into wells 2 through 11 of row E to serve as the background readings. Plates were incubated for one hour in the dark before reading. Fluorescence reading was measured at

485 nm (excitation) and 528 nm (emission) using the BMG labtech fluostar optima analyzer. Fluorescence readings were plotted against haematocrit to determine relative fluorescence effect.

3.2.3 Relationship between Sybr Green fluorescence and extracted parasite DNA

Saponin (10%) in PBS was used to lyse red blood cells while leaving the *Plasmodium falciparum* parasite intact with its parasite membrane and parasitophorous vacuole membrane for genomic DNA extraction. Twelve milliliters (12 ml) of 3D7 *P. falciparum* culture at 4% hematocrit and 5% parasitaemia was centrifuged at 1400 rpm for 3 minutes after which media was aspirated. Aliquots of red cells pellets were suspended in 1 ml of 10% Saponin in 1.5 ml eppendorf tubes and incubate for 5 minutes on ice. The process was then followed by washing with 1ml 1×PBS by centrifugation at 6000 rpm for 3 minutes followed by removal of the supernatant leaving dark pellets of parasite. The dark pellets were re-suspending in 1ml 1×PBS and washing process was repeated 3 to 4 times and then, the pellets were lysed with lysing buffer which consisted of Tris 20mM [pH 7.5], EDTA [5mM], saponin [0.008%; wt/vol], and Triton X-100 [0.08%; wt/vol]) to release the DNA. The extracted DNA was suspended in 100 µl of DEPC nuclease free water (BioExpress), and its genomic content was quantified using a ND-1000 NanoDrop® (NanoDrop Technologies, Inc.), before being stored at −20°C. 200µl of the extracted DNA was put into wells of column 2 through 11 of row A, B, C of the 96-well microtitre plate in triplicate and then100µl of culture media was also put into the wells of column 2 through to 11 of row A, B and C. Two-fold serial dilution was performed starting from wells of column 2 through 11 of row A, B, C to obtain a concentration range of 0.2ng/µl to 0.003 ng/µl of the DNA. 100µl of the lysis buffer and sybr green mixture were added to 100µl of PBS into wells 2 through 11 of row D to serve as the background readings. Fluorescence

reading was measured at 485 nm (excitation) and 528 nm (emission) using the BMG labtech fluostar optima analyzer.

3.3 In vitro drug sensitivity testing of *P. falciparum* field isolates using the Sybr Green method.

3.3.1 Collection of P. falciparum field isolates

P. falciparum field isolates used for the in vitro test was obtained from an ongoing drug monitoring study being carried out by the Noguchi memorial Institute for Medical research in three sentinel sites in Ghana in collaboration with the National Malaria control Program. The Sentinels sites are Hohoe, Navrongo and Cape Coast.

The study population consisted of children with uncomplicated *P. falciparum* malaria attending a study clinic and participation consent was obtained from the children's parents or guardians. The following were set of inclusion criteria that were adhered to. Ages between 0-59 months, mono-infection with *P. falciparum* detected by microscopy, asexual parasitaemia of 1,000-250,000/ µl, presence of axillary temperature ≥ 37.5°C or history of fever during the past 24 hours. Blood samples were collected into vacutainer tubes which already contained 0.75ml of acid citrate dextrose solution (ACD).

3.3.2 Drug solution preparation

Stock concentration; 1mg/ 1ml

Drug solutions were prepared by dissolving 5mg of chloroquine (CQ) in 2.38ml of 70% ethanol and sonicated to completely dissolve the drug and the volume is made up to 5ml with 2.62ml distilled water giving a stock concentration of 1mg/1ml (1000000ng/ml). 5mg of artesunate (AS) was dissolved in 5ml of Dimethyl sulfoxide (DMSO) of stock concentration 1mg/1ml (1000000ng/ml) and sonicated to completely dissolve the drug. 1µl

of artesunate drug to 4999µl of 10% RPMI culture media was used in diluting the drug to a Starting concentration of 200ng/ml. 10µl of Chloroquine was diluted in 4990µl of the 10% culture media to a Starting concentration of 2000ng/ml.

3.3.3 Preparation of Chloroquine (CQ) and Artesunate (AS) templates.

A 96- well sterile microtitre plate was opened in a laminar flow hood and 200µl of Chloroquine solution of concentration 2000ng/ml was put into wells of column 1 rows , B, C, D and 200µl of Artesunate of concentration 200ng/ml into wells of column 1 row E, F, G as shown in fig. 2. Hundred microliters (100µl) of incomplete culture media was transferred into the remaining wells of column 2 to 10 of row B, C, D, E, F, and G. A 2-fold serial dilution of each drug was performed by transferring 100µl of the content of each well from column 1 row A to G through to column 10. Contents in each well were mixed with a multichannel pipette before transfer. 12.5µl of drug solution after the serial dilution described above from each well was transferred into the wells of column 2 to 10 of row B, C, D, E, F and G of a new sterile microtitre plate to setup the test plate (pre-dosed plate). The wells of column 11 of row B, C, D, E, F and G served as the control (no drug wells). Row H served as the background control (no parasites wells).

3.3.4 In vitro assay with fresh clinical isolates

Two milliliters (2ml) of venous blood after establishing positive parasitaemia (asexual parasitaemia of 1,000-250,000/µl) from thick smear was collected into vacutainer tubes with 0.75ml acid citrate dextrose solution (ACD) anticoagulant. One milliter (1ml) of the 2ml blood (normal human blood haematocrit ranges from 40%-45%) samples collected from patients was diluted with 20ml of parasite culture media (CPM) to give a haematocrit

of 2%. Hundred microliters (100µl) of this preparation was then dispensed into the test plate (pre-dosed plates). Column 11 and row H are the Positive and negative controls respectively. Positive control wells contained only the infected blood without the drug solution while the negative control wells contained uninfected blood as the blank. Each drug was tested in triplicate. The plates were then gassed for two 20 seconds with 5.5% CO_2/balanced N_2 and 2% O_2 gas mixture. Plates were incubated at 37°C for 72hrs after which it was frozen at -20°C until ready for processing for fluorescence reading.

3.3.5 Determination of IC_{50} by fluorescence.

Following incubation and freezing, the plates were thawed for 2 hours at room temperature and each sample mixed with a multichannel pipette. Hundred microliters (100µl) of the lysis buffer-sybr green I mixture (final concentration, 1/10,000 [Molecular Probes, Invitrogen, Carlsbad, CA] which consisted of Tris 20mM [pH 7.5], EDTA [5mM], saponin [0.008%; wt/vol], and Triton X-100 [0.08%; wt/vol]) was added to the 112.5µl content of each well of rows B and F of the test plate (pre-dosed plates). The plates were then covered with a foil and incubated at room temperature for 1hour. Fluorescence readings was measured at 485 nm (excitation) and 528 nm (emission) using the BMG labtech fluostar optima analyzer. The Sybr green-1 assay generates fluorescence arbitrary values at various concentrations of the parasite DNA as raw data. Fluorescence values from positive control wells represent the maximum fluorescence that is produced by the amount of parasite DNA and fluorescence values from blank wells (parasite-free red blood cells) represent background fluorescence. The 100% growth value was obtained by subtracting the mean fluorescence value of blank wells from that of positive control wells (drug-free wells). The growth value at each concentration of the drug was obtained by subtracting fluorescence values of drug-treated wells from positive control wells. These

values were then expressed as a percentage of the 100% growth value. Fluorescence values were plotted against corresponding concentrations of the drug to determine the 50% inhibition of the parasites (IC_{50}) using the antimalaria research network (WWARN) (Le Negard *et al.,* 2010) to generate dose-response curves from which IC_{50} values were obtained. The different IC_{50s} values obtained were then grouped and their means calculated and compared among themselves using Independent-Samples *t*-test.

3.3.6 Determination of IC_{50} by microscopy

Lysate from each well of row D with chloroquine and row E with artesunate of the test plate (pre-dosed plates) after carefully discarding the supernatant were spread per slide to make a thick smear. The smears were air dried and stained with 10% Giemsa solution for 10-15 minutes. All slides were examined microscopically and the numbers of schizonts per field were counted in the thick film under oil immersion using the 100x magnification. The total numbers of white blood cells (WBC) were also counted per field in the thick film under oil immersion using the 100x magnification. Number of schizonts was counted against 200 WBC for each well of row D and E of a plate. The growth value at each concentration of the drug was obtained by subtracting values of drug-treated wells from wells with parasites but without the drug (positive control wells). These values were then expressed as the 100% growth value. Schizont count values were plotted against corresponding concentrations of the drug to determine the 50% inhibition of the parasites (IC_{50}) using the antimalaria research network (WWARN) (Le Negard *et al.,* 2010) to generate dose-response curves from which IC_{50} values were obtained. The different IC_{50}s values obtained were then grouped and their means calculated and compared among themselves using Independent-Samples *t*-test.

3.4 Statistical Analysis

GenStart 9th Edition statistical package was used for regression and analysis of variance (ANOVA). Graphpad prism software package was used for graphical presentation of data. Fifty percent inhibition concentration (IC_{50}) values were determined from the dose response data by nonlinear regression analysis of the plot of drug concentration as relative effect against fluorescence reading using the online antimalaria research network (WWARN) (Le Negard *et al.*, 2010).

CHAPTER FOUR

4.0 ANALYSIS OF TEST RESULTS FROM THE SYBR GREEN-1 ASSAY

4.1 Relationship between fluorescence of Sybr Green and level of parasitaemia at different incubation periods.

The sybr green fluorescence is dependent on the concentration of parasite DNA that binds to the dye (Smilkstein *et al*., 2004). Fluorescence was assessed in both instances of adding sybr green dye before incubation and freezing the samples before the addition of the dye. The Sybr green-1 assay generates fluorescence arbitrary unit (A.U) values at various concentrations of the parasite DNA as raw data. Fluorescence values from test wells represent the maximum fluorescence that is produced by the amount of parasite DNA and fluorescence values from blank wells (parasite-free red blood cells) represent background fluorescence. Maximum fluorescence was obtained by subtracting the mean fluorescence value of blank wells from fluorescence values of the test wells.

There was no difference (p= 0.0.988) in fluorescence for 5min incubation and 1hr incubation. Fluorescence rates of 24.3% (56114/232354.3) for 1hr incubation and 20.3% (58958.7/290207) for 3hrs incubation (table 1) showed a significant difference (F=1.35 and p=0.257) with a coefficient of variation of 65.8 % and 5617.8 standard error of difference of means (s.e.d). There was also a significant difference (F=1.19 and P=0.286) between fluorescence reading for incubating the samples overnight as against fluorescence readings for five minutes incubation after the addition of sybr green and lysis buffer mix. In two separate experiments, the difference between Fluorescence rates 13.3% (59877.7/4497123) and 24.3% (56114/232354.3) (table 1) respectively for freezing samples for 1hr before the addition of sybr green and lysis buffer and incubating the

samples 1hr after the addition of sybr green and lysis buffer mix was significant (F=3.42 and P=0.078).

The relationship between the levels of parasitaemia and the fluorescence units determined after the addition of sybr green and lysis buffer mix incubated in the dark, showed a well-correlated linear relationship (r^2 =0.9962) as well as the fluorescence readings for freezing the samples for one hour before the addition of sybr green and lysis buffer mix (r^2 = 0.9447) (figure 3). The quantification limit (QL) as determined by the assay in the test wells was 0.5%. The experiment also produced a z'- value of ≥4.7 for a test well which is well within a quality assay acceptable range.

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Table 1. *Fluorescence units at different incubation time periods.*

(%) Parasitaemia	Mean fluorescence(FL) Arbitrary unit (A.U) for five min	Mean FL (A.U) for 1hr Of incubation of samples.	Mean FL (A.U)for 3hrs Of incubation of samples	Mean FL (A.U) after incubation of samples overnight	Mean FL (A.U) after freezing of samples for 1hr
7.8	56114	56114	58958.7	59412	59877.7
3.9	33807.3	33807.3	41089	40867.3	45860.3
1.95	22564	22564	31082.7	30921.3	36024.3
0.975	17484	17484	21450.7	21687	32832.7
0.4875	14091.3	15091.3	23377.7	22435.7	28063
0.24375	12716	12716	17384.7	17236	23913.7
0.121875	12412.7	12412.7	17153	16960.3	21534.3
0.060938	14693.3	14693.3	16655	16074.7	20349.3
0.030469	11513	11513	15691.7	15101.7	20917.3
0.015234	11703.3	11703.3	15776.3	15121	20395
0.007617	11857.7	11857.7	15811.3	14819.7	19763.7
0.003809	12397.7	12397.7	15776.2	15517	20181

The relationship between the levels of parasitaemia and fluorescence at different incubation periods after the addition of sybr green and lysis buffer mix. Two-fold serial dilution of 3D7 culture of initial parasitaemia: 7.8% in uninfected blood and medium mixture was performed. Incubation was done in the dark after the addition of sybr green/lysis buffer mixture. Background fluorescence, defined as fluorescence detected in

the absence of DNA, was subtracted from each data point. Two percent (2%) haematocrit blood was used in the experiment. Fluorescence is measured as arbitrary units (A.U.). Data shown are the mean of 3 independent determinations.

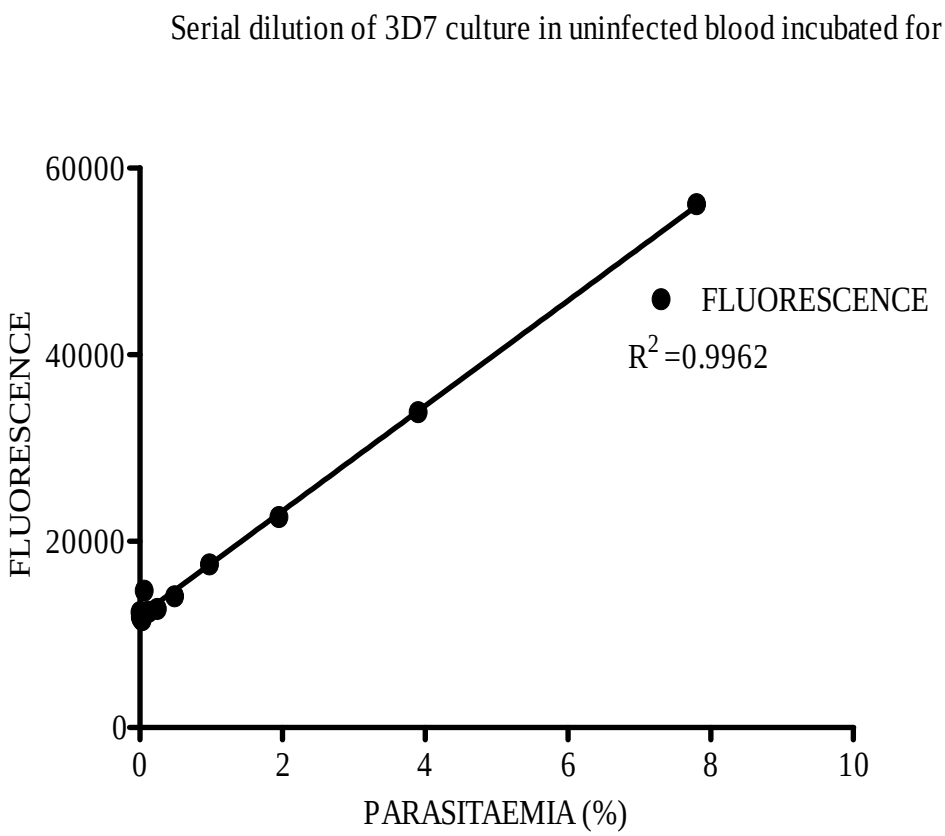

Fig. 4. The relationship between the levels of parasitaemia and fluorescence at 5min incubation period after the addition of sybr green and lysis buffer mix. Two-fold serial dilution of 3D7 culture of initial parasitaemia: 7.8% in uninfected blood and medium mixture was performed. Incubation was done in the dark after the addition of sybr green/lysis buffer mixture. Background fluorescence, defined as fluorescence detected in the absence of DNA, was subtracted from each data point. Two percent (2%) haematocrit blood was used in the experiment. Fluorescence is measured as arbitrary units (A.U.). Data shown are the mean of 3 independent determinations

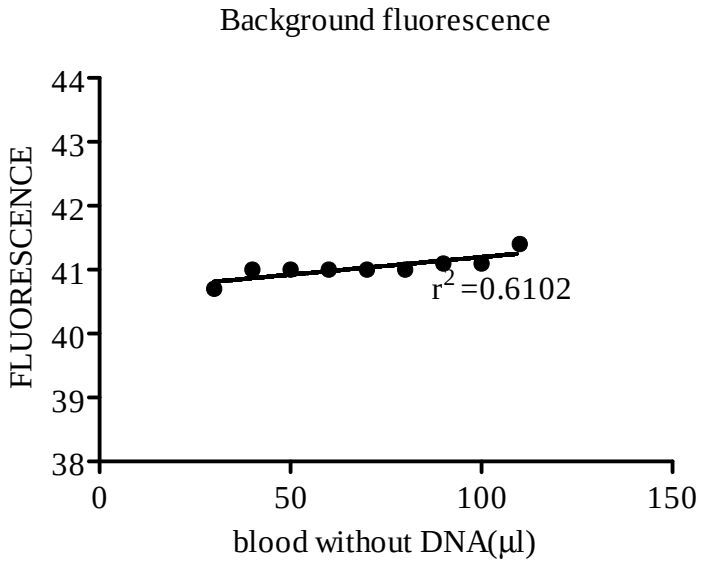

Fig. 5. Background fluorescence detected in the absence of DNA. Fluorescence is measured as arbitrary units (A.U.) at 485nm excitation and 528nm emission.

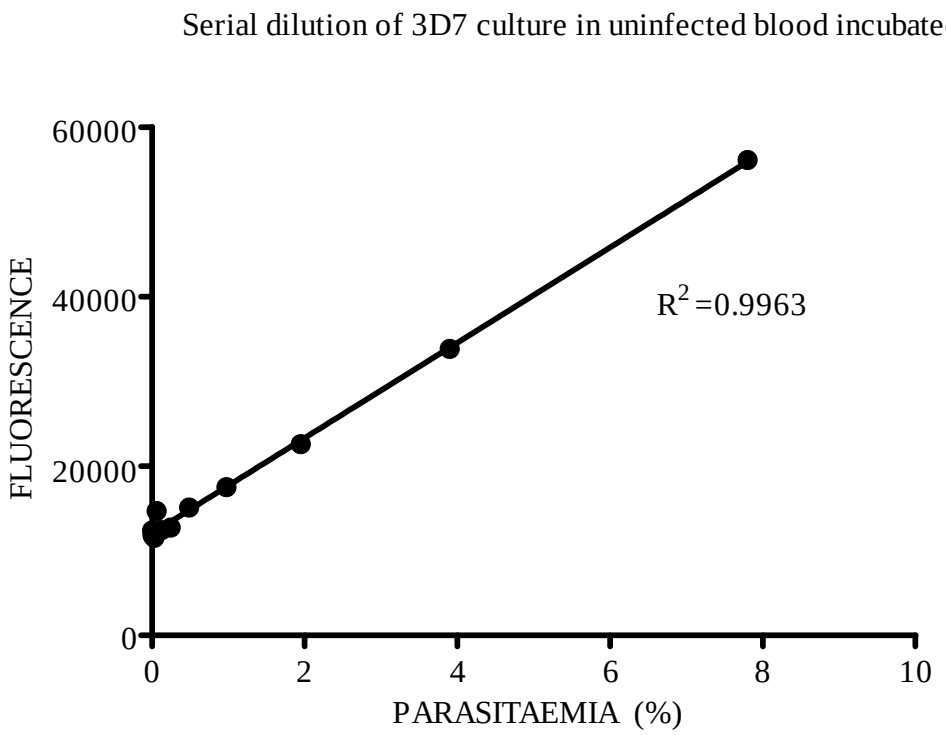

Fig. 6. The relationship between the levels of parasitaemia and fluorescence at 1hr incubation period after the addition of sybr green and lysis buffer mix. Two-fold serial dilution of 3D7 culture of initial parasitaemia: 7.8% in uninfected blood and medium mixture was performed. Background fluorescence, defined as fluorescence detected in the absence of DNA, was subtracted from each data point. Two percent (2%) haematocrit blood was used in the experiment. Fluorescence is measured as arbitrary units (A.U.). Data shown are the mean of 3 independent determinations.

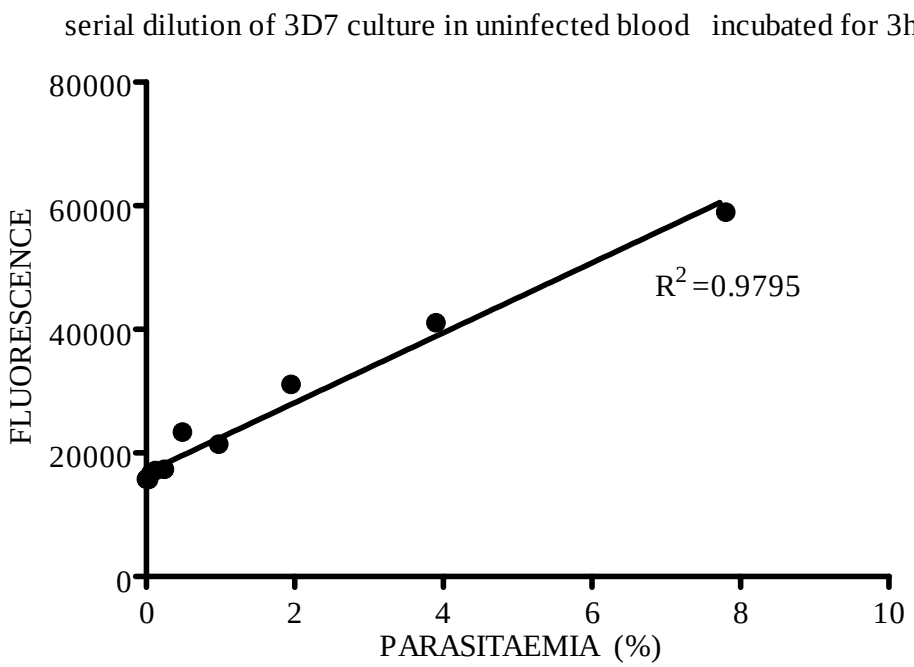

Fig. 7. The relationship between the levels of parasitaemia and fluorescence at 3hr incubation period after the addition of sybr green and lysis buffer mix. Two-fold serial dilution of 3D7 culture of initial parasitaemia: 7.8% in uninfected blood and medium mixture was performed. Background fluorescence, defined as fluorescence detected in the absence of DNA, was subtracted from each data point. Two percent (2%) haematocrit blood was used in the experiment. Fluorescence is measured as arbitrary units (A.U.). Data shown are the mean of 3 independent determinations. A Statistical difference in fluorescence (F=1.35 and P= 0.257) was observed when compared with 5min incubation of the samples.

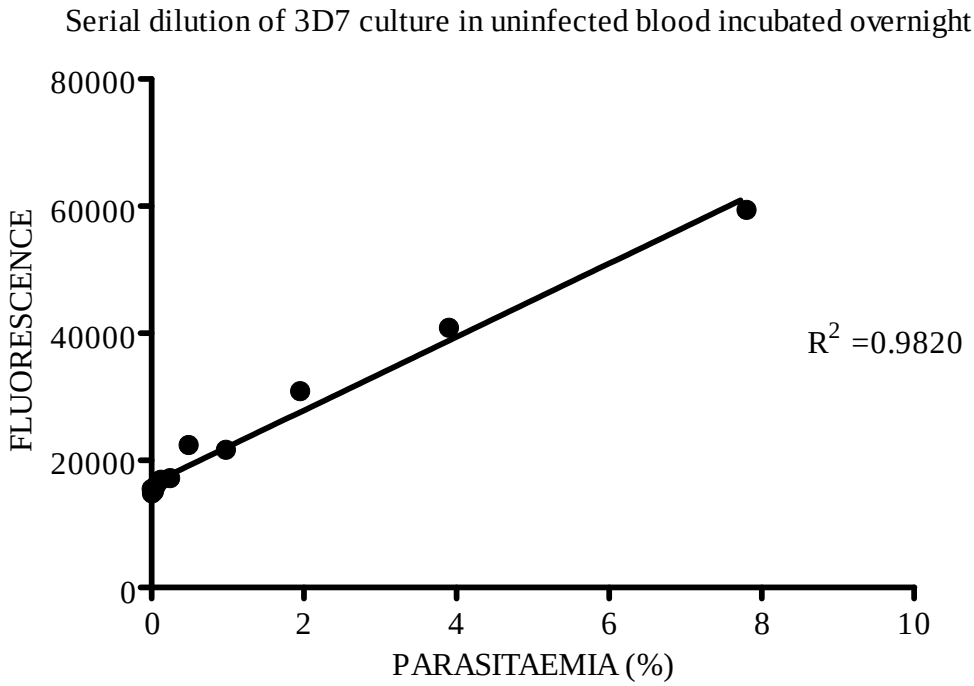

Fig. 8. The relationship between the levels of parasitaemia and fluorescence at overnight incubation period after the addition of sybr green and lysis buffer mix. Two-fold serial dilution of 3D7 culture of initial parasitaemia: 7.8% in uninfected blood and medium mixture was performed. Background fluorescence, defined as fluorescence detected in the absence of DNA, was subtracted from each data point. Two percent (2%) haematocrit blood was used in the experiment. Fluorescence is measured as arbitrary units (A.U.). Data shown are the mean of 3 independent determinations. A Statistical difference (F=1.19 and P=0.286) was observed when compared with 5min incubation of the samples.

Serial dilution of 3D7 culture in uninfected blood for 1hr freezing.

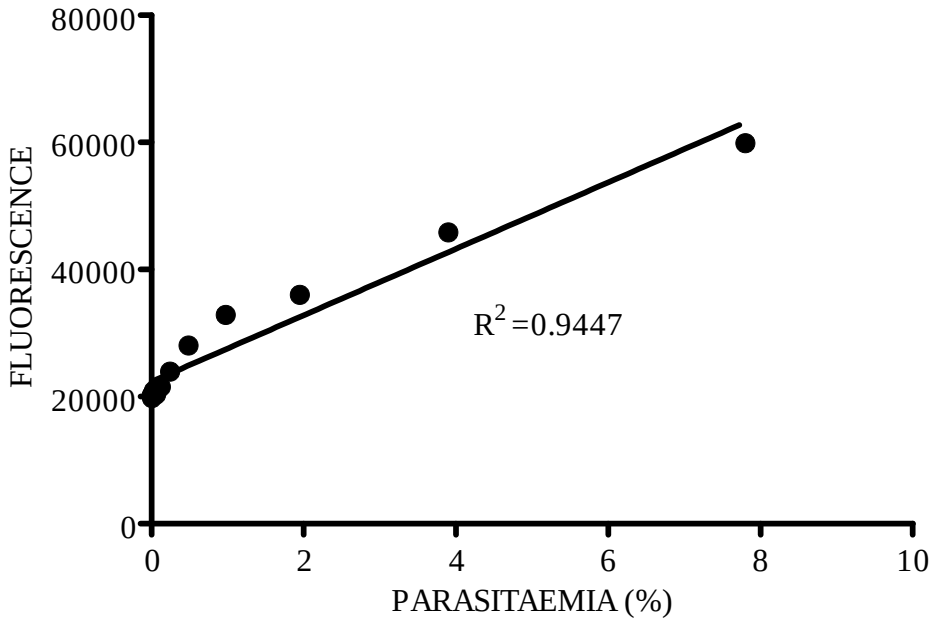

Fig. 9. The relationship between the levels of parasitaemia and fluorescence for freezing samples for 1hr before the addition of sybr green and lysis buffer mix. Two-fold serial dilution of 3D7culture of initial parasitaemia: 7.8% in uninfected blood and medium mixture was performed. Background fluorescence, defined as fluorescence detected in the absence of DNA, was subtracted from each data point. Two percent (2%) haematocrit blood was used in the experiment. Fluorescence is measured as arbitrary units (A.U.). Data shown are the mean of 3 independent determinations. Fluorescence difference (F=3.42 and P=0.078) was observed when compared with 1hr incubation of the samples.

4.2 Assessing sybr green fluorescence with varying haematocrit (HCT) at fixed salmon sperm DNA

The effect of haematocrit on sybr green fluorescence was investigated. Haematocrit was varied from 5% to 0.3% with a constant salmon sperm DNA concentration of 1000ng/ml. The results of this experiment showed that Sybr green fluorescence decreases as haematocrit increases (Table 2) with a positive linear correlation (R^2=0.9492) in figure 9. There was a poor correlation in fluorescence with 1x PBS without salmon sperm DNA.

Table 2. *Outcome of experiment to demonstrate the effect of haemoglobin on sybr green fluorescence at varying haematocrit (HCT).*

HCT (%)	Fluorescence unit (A.U)
5	48.75
4	54.02
3	130.62
2	144.42
1	185.93
0.5	234.91
0.4	227.74
0.3	262.3

Sybr green fluorescence with varying haematocrit at a constant salmon sperm DNA (1000ng/ml) concentration. Fluorescence was measured as arbitrary units (A.U.) at 485nm (excitation) and 528nm (emission).

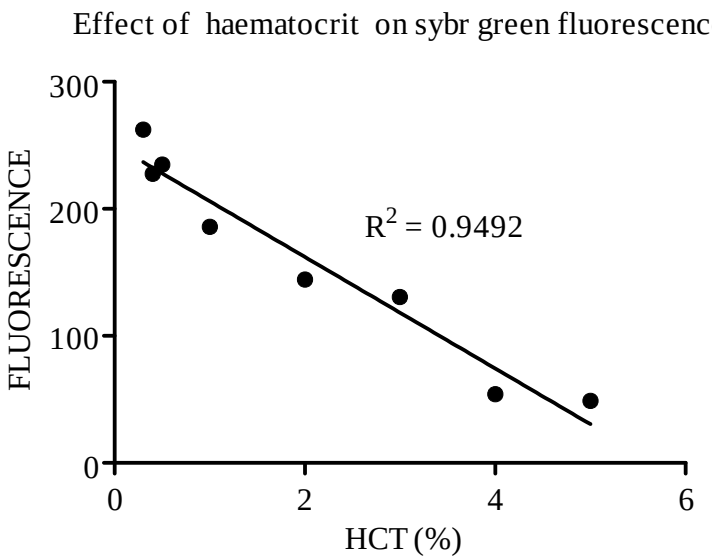

Fig 10. Relationship between Sybr green fluorescence and varying haematocrit at constant salmon sperm DNA concentration (1000ng/ml). Fluorescence is measured as arbitrary units (A.U.) at 485nm excitation and 528nm emission.

4. 3. Assessing sybr green fluorescence with extracted parasite DNA at varying concentrations.

Upon treatment of infected blood with 10% saponin and washing in 1x PBS to remove all the haemoglobin, the parasite DNA was extracted with lysing buffer. Two-fold serial dilution of the extracted 3D7 parasite DNA (0.2ng/µl) at 5% initial parasitaemia indicated an increasing linear trend (Table 3) with Sybr green fluorescence.

Table 3. *Outcome of the experiment involving extracted DNA showing Fluorescence at varying DNA concentrations.*

Extracted DNA (ng/µl)	Fluorescence unit (A.U)
0.2	308.84
0.1	167.08
0.05	89.95
0.025	59.66
0.0125	50.35
0.00625	46.39
0.003125	41.89

Sybr green fluorescence with varying extracted parasite DNA at 0.2ng/µl (200ng/ml). Fluorescence was measured as arbitrary units (A.U.) at 485 nm (excitation) and 528 nm (emission).

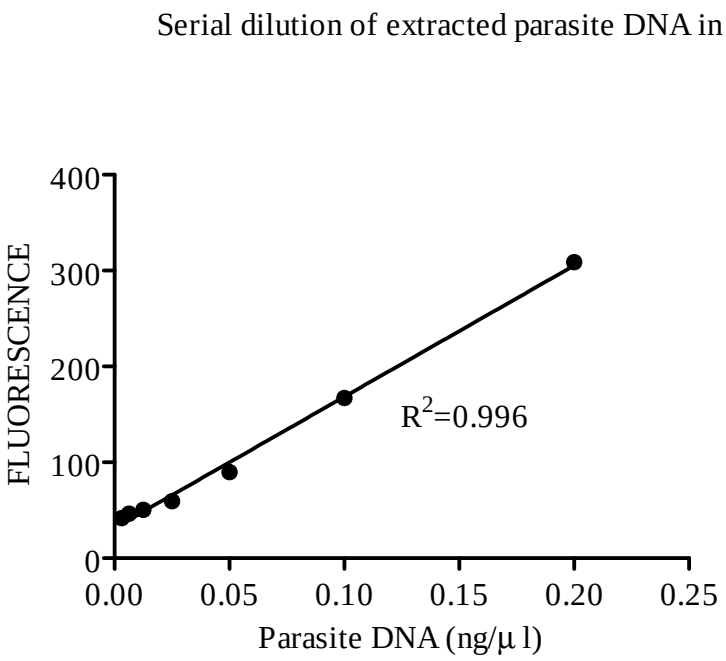

Fig 11. Relationship between Sybr green fluorescence and extracted parasite DNA. Two-fold serial dilution of extracted 3D7 DNA at 0.2ng/µl (200ng/ml) initial concentration in PBS was performed. Fluorescence is measured as arbitrary units (A.U.) at 485nm excitation and 528nm emission.

4.4 In vitro sensitivity of *P. falciparum* to artesunate and chloroquine

In vitro drug sensitivity of *P. falciparum* field isolates to artesunate (AS) and chloroquine (CQ) assessed in experiment involving sybr green assay and microscopy methods were similar. Isolates were subjected to chloroquine and artesunate for the 72hrs life cycle of *P .falciparum*.

Representative graphs, illustrating the response of *P .falciparum* field isolates strain to chloroquine and artesunate in one experiment, are shown (Fig.12 & 13). All successfully tested parasite isolates were sensitive to artesunate and chloroquine after subjecting isolates to chloroquine and artesunate two-fold serial dilution dose. Analyses from the test and control wells indicated a percentage growth of 63% and 37% for chloroquine and artesunate respectively. IC_{50} values were well below the IC_{50} threshold for resistance (table 4&5). 100nM (Samuel *et al.,* 2010) is the IC_{50} threshold for resistance to chloroquine. Studies on sensitivity cut-off point for artesunate are not conclusive but an IC_{50} of 20nM is considered high (Ferreira *et al*., 2007). The Correlations of antiplasmodial activity of chloroquine against *P*. falciparum in this experiment assessed by microscopy and sybr green assay methods was (r^2=0.1182) with a mean IC_{50} of 0.013nM and 3.17nM (P=0.1378, CI;-2.563 to 17.10nM) respectively by both methods. Antiplasmodial activity of artesunate against *P*. falciparum assessed by microscopy and sybr green assay methods indicated a Correlation of (r^2 =0.0098) with mean IC_{50} of 0.01nM and 0.29nM (P=0.6772, CI; -21.38 to 14.21) respectively by both methods.

4.4.1 Determination of IC_{50} from dose-response curve.

Figure12 (a&b) and figure13 (a&b) shows the maximal drug effect on the parasites. It is a representation of one sample out of a total of 20 samples. The IC50s of the 20 sample are presented in (table 4 and 5).

P. falciparum clinical isolates response to Chloroquine obtained from schizont count in thick smear.

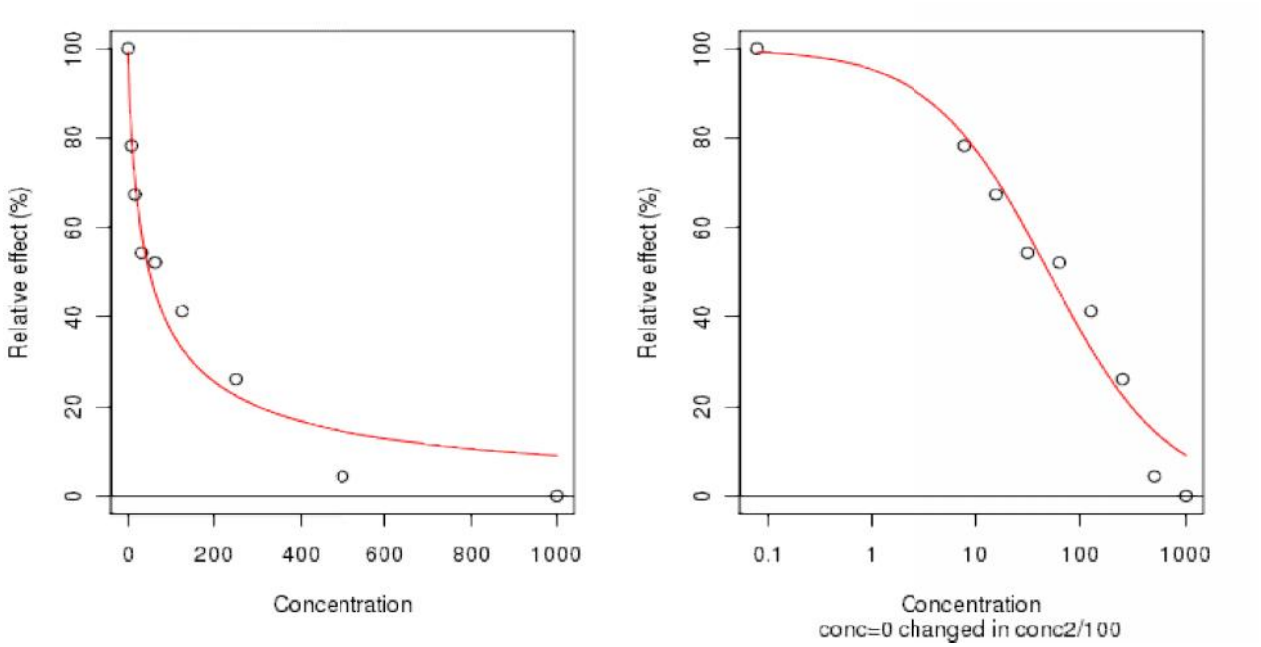

Fig 12a, : Dose-response curve showing the relationship between the levels of drug concentrations and relative effect. Two-fold serial dilution of chloroquine of initial concentration 2000ng/ml in culture medium was performed in wells of a 96-well microtitre plate to set up a pre-dose plate. parasite isolates were then subjected to the drug in the pre-dose plate. The culture was kept at 37°C under a gas mixture of 2% O_2, 5.5% CO_2, and 92.5% N_2 for a full *P. falciparum* cycle of 72hrs. The number of parasites that survived in each drug well after incubation were counted against 200 WBCs in thick smear using microscopy and represented as the relative effect

on the the graph. The meeting points on the curve of a projection from 50% relative effect to the curve on the second graph to the right marks the corresponding the drug concentration(IC_{50}) that inhibites 50% of the parasites.

P. falciparum clinical isolates response to artesunate obtained from schizont count in thick smear.

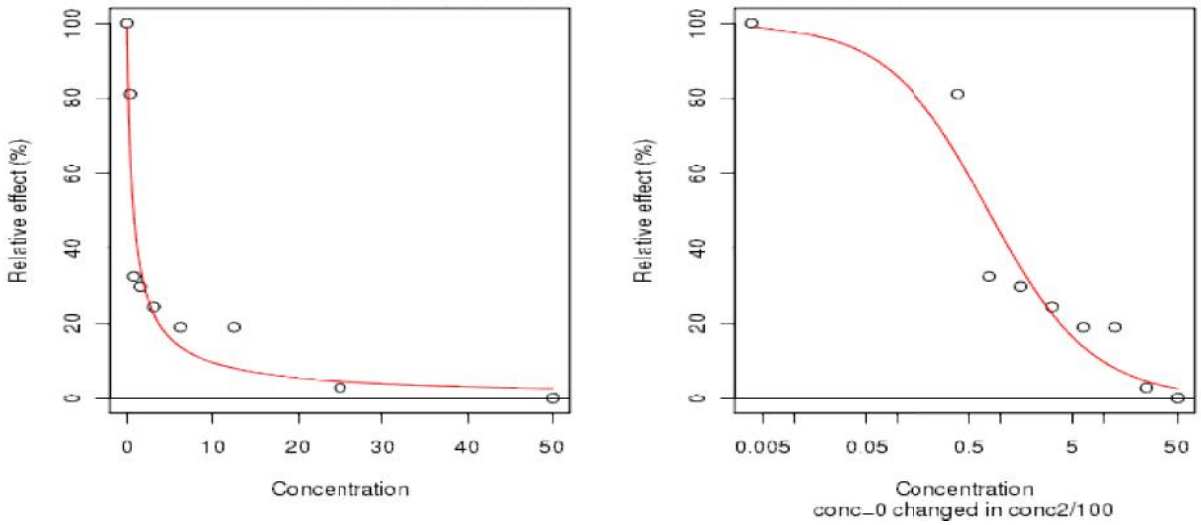

Fig 12b. Dose-response curve showing the relationship between the levels of drug concentrations and relative effect. Two-fold serial dilution of chloroquine of initial concentration 2000ng/ml in culture medium mixture was performed in wells of a 96-well microtitre plate to set up a pre-dose plate. parasite isolates were then subjected to the drug in the pre-dose plate. The culture was kept at 37°C under a gas mixture of 2% O_2, 5.5% CO_2, and 92.5% N_2 for a full *P. falciparum* cycle of 72hrs. The number of parasites that survived in each drug well after incubation were counted against 200 WBCs in thick smear using microscopy and represented as the relative effect on the the graph. The meeting points on the curve of a projection from 50% relative effect to the curve on the second graph to the right marks the corresponding the drug concentration(IC_{50}) that inhibites 50% of the parasites.

Table 4*.Outcome of the In vitro testing of the susceptibility of P. falciparum field isolates to artesunate (AS) and chloroquine (CQ) using the microscopy method, N=20*

Sample No	Drug				
	CQ	IC_{50}nM (CI)	S.E	AS	IC_{50}nM (CI) S.E
1	0.001(0.29-0.45)	0.03	0.467(66-196.4)	26.48	
2	0.002(0.58-0.78)	0.04	0.225(45.86-81.19)	6.87	
3	0.004(0.97-1.43)	0.1	0.027(6.36-8.69)	0.51	
4	0.004(0.98-1.49)	0.11	0.026(5.98-8.7)	0.57	
5	0.002(0.59-0.87)	0.06	0.027(5.86-9.49)	0.8	
6	0.004(1.02-1.443)	0.09	0.269(47.27-104.51)	12.1	
7	0.023(6.26-8.58)	0.49	0.019(4.89-6.22)	0.21	
8	0.287(63.9-119.46)	11.75	0.001(0.27-0.39)	0.03	
9	0.022(5.93-8.35)	0.51	0.004(1.01-1.32)	0.06	
10	0.023(5.97-8.66)	0.55	0.003(0.6-0.8)	0.04	
11	0.01(2.66-3.92)	0.27	0.003(0.58-0.87)	0.06	
12	0.02(6.02-8.32)	0.27	0.003(0.58-0.87)	0.07	
13	0.02(5.79-8.91)	0.66	0.003(0.57-0.88)	0.07	
14	0.01(3.09-4.61)	0.32	0.004(0.95-1.5)	0.11	
15	0.10(20.54-45.42)	0.26	0.014(2.79-4.96)	0.44	
16	0.12(23.26-57.21)	5.91	0.001(1.24-2.04)	0.16	
17	0.02(6.02-8.32)	0.47	0.003(0.55-0.89)	0.07	
18	0.02(5.79-8.91)	0.66	0.003(0.57-0.88)	0.007	
19	0.01(3.09-4.61)	0.32	0.004(0.95-1.5)	0.11	
20	0.01(0.66-3.92)	0.27	0.003(0.58-0.87)	0.06	

N: number of parasites isolates tested. CI: confidence intervals are given in the bracket. S.E: standard error. CQ: chloroquine. AS: artesunate. IC_{50}: 50% concentrations (half of the drug concentration that has maximal effect). The IC_{50} value of each sample was obtained

from dose response curve. The concentration (IC_{50}) of the drug that exhibits 50% of the parasites after the full *P. falciparum* cycle of 72hrs of incubation were obtained in ng/ml and then converted from ng/ml to nM given against the confidence intervals in the bracket.

P. falciparum clinical isolates response to chloroquine obtained from fluorometric reading (sybr green assay)

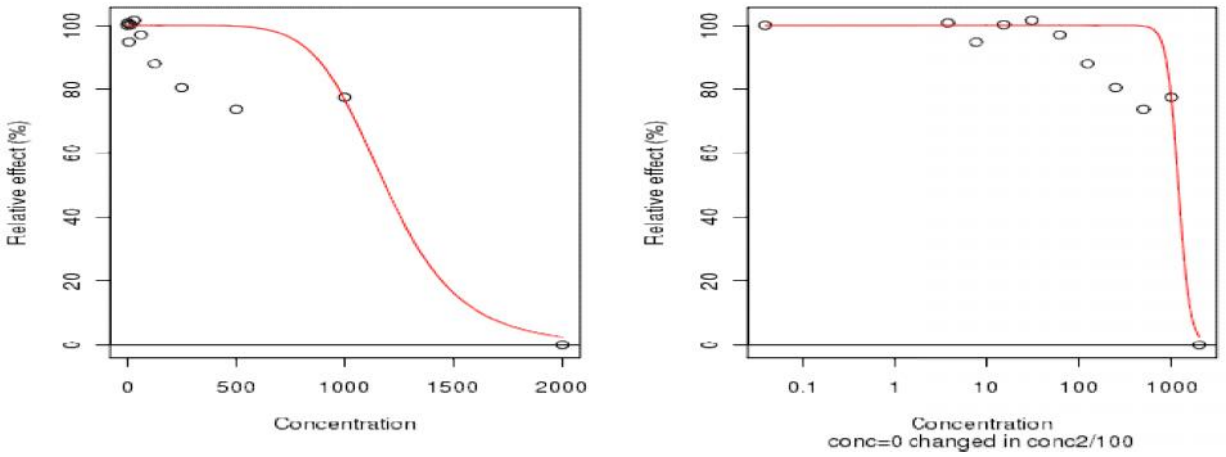

Fig 13a: Dose-response curve showing the relationship between the levels of drug concentrations and relative effect. Two-fold serial dilution of artesunate of initial concentration 2000ng/ml in culture medium mixture was performed in wells of a 96-well microtitre plate to set up a pre-dose plate. parasite isolates were then subjected to the drug in the pre-dose plate. The culture was kept at 37°C under a gas mixture of 2% O_2, 5.5% CO_2, and 92.5% N_2 for a full *P. falciparum* cycle of 72hrs. The number of parasites that survived in each drug well after incubation were measured in the form of fluorescence units and represented as the relative effect on the the graph. The meeting point on the curve of a projection from 50% relative effect to the curve on the second graph to the left marks the corresponding concentration (IC_{50}) of the drug that inhibits 50% of the parasites

P. falciparum clinical isolates response to artesunate obtained from fluorometric reading (sybr green assay)

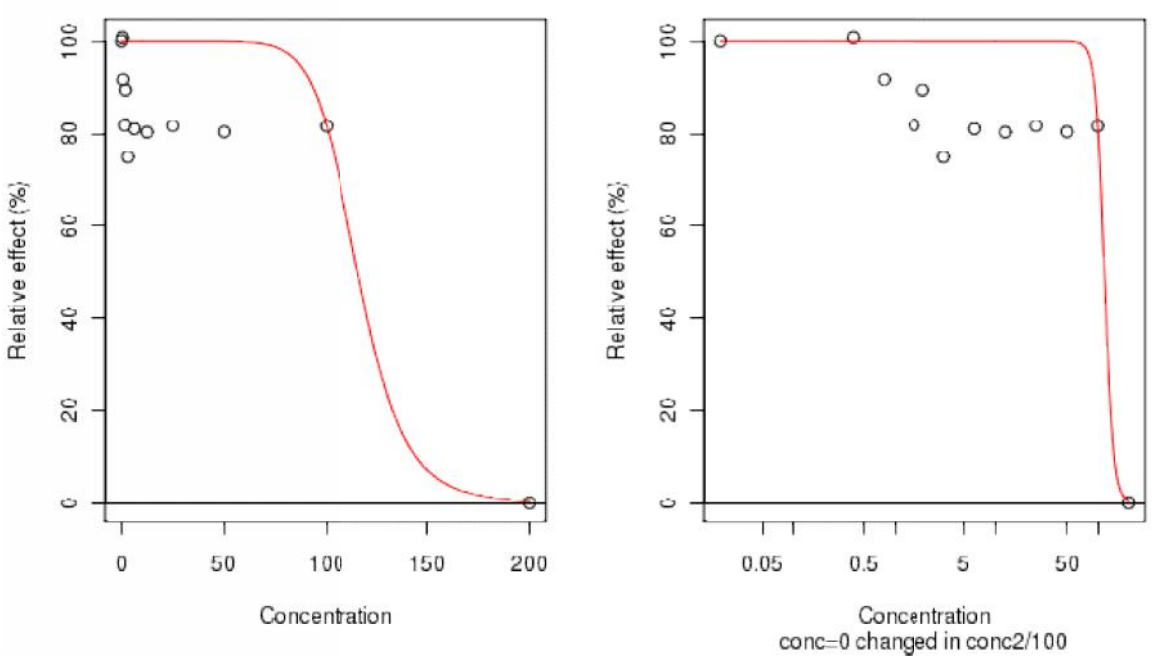

Fig 13b: Dose-response curve showing the relationship between the levels of drug concentrations and relative effect. Two-fold serial dilution of artesunate of initial concentration 2000ng/ml in culture medium mixture was performed in wells of a 96-well microtitre plate to set up a pre-dose plate. parasite isolates were then subjected to the drug in the pre-dose plate. The culture was kept at 37°C under a gas mixture of 2% O_2, 5.5% CO_2, and 92.5% N_2 for a full *P. falciparum* cycle of 72hrs. The number of parasites that survived in each drug well after incubation were measured in the form of fluorescence units and represented as the relative effect on the the graph. The meeting point on the curve of a projection from 50% relative effect to the curve on the second graph to the left marks the corresponding the concentration (IC_{50}) of the drug that inhibites 50% of the parasites.

IC50s of 20 samples showing the maximal drug effect on the parasites.

Table 5. *Outcome of the In vitro testing of the susceptibility of P. falciparum field isolates to artesunate (AS) and chloroquine (CQ) using the sybr green assay, N=20*

Sample No	Drug				
	CQ	IC_{50}nM (CI)	S.E	AS IC_{50}nM (CI)	S.E
1	3.42 (4.48-2179.37)		480.71	0.001(0.2-0.6)	0.09
2	4.708(1094-1913)		185	0.555(39.88-272.49)	2.84
3	4.950(-968-969)		435	17.804(67.09-213.29)	33.21
4	4.459(888-1960)		240	0.524(97.6-197)	22.48
5	4.82(2.53-3077.94)		690.13	0.005(0.64-2.09)	0.32
6	3.782(1002-1413)		90.86	0.529(42.14-255)	47.97
7	6.244(550-1839)		289	0.484(-203.2-476.16)	152.45
8	4.838(647-2444)		412	0.534(64.25-236.74)	39.58
9	3.753(1005-1392)		86.79	0.020(1.17-10.23)	2.06
10	0.763(135.93-351.82)		49.04	0.310(58.69-139.7)	18.07
11	4.657(1033-1451)		93.8	0.490(-120-407)	118.48
12	3.888(697-1675)		216	0.508(89.89-142.27)	11.9
13	3.173(847-2221)		308	0.412(14.92-32.71)	4.04
14	0.739(989-2006)		228	0.08(-23.15-293.6)	71.97
15	4.804(714-2256)		340	0.531(0.2-0.6)	0.09
16	4.688(26.42-91.78)		14.67	0.480(66.47-96.41)	6.72
17	4.648(714-2256)		340	0.497(-55-377)	98.89
18	0.185(26.42-91.78)		14.67	0.289(66.47-96.41)	6.72
19	4.950 (-686.61-3849.78)		1033.54	10.567(-57-377)	98.89
20	4.608(789-2155)		306.56	0.528(79.79-217.77)	30.96

N: number of parasites isolates tested. CI: confidence intervals are given in the bracket. S.E: standard error. CQ: chloroquine. AS: artesunate. IC_{50}: 50% inhibitory concentrations

(half of the drug concentration that has maximal effect). The IC_{50} value of each sample was obtained from dose response curve. The concentration (IC_{50}) of the drug that inhibites 50% of the parasites after the full *P. falciparum* cycle of 72hrs of incubation were obtained in ng/ml and then converted from ng/ml to nM given against the confidence intervals in the bracket.

Table 6. Correlation of in vitro susceptibility of *P. falciparum* field isolates to chloroquine and artesunate determined by microscopy and sybr green assay methods.

Method	Drug; Chloroquine: Isolates n=20			
Microscopy	IC_{50}(nM) mean 0.013	r^2	95% CI	P-value
		0.1182	-2.563 – 17.10	0.1378
Sybr Green-1	IC_{50}(nM) mean 3.17			
	Drug; Artesunate: Isolates n=20			
Microscopy	IC_{50}(nM) mean 0.01	r^2	95% CI	P-value
		0.0098	-21.38 – 14.21	0.6772
Sybr Green-1	IC_{50}(nM) mean 0.29			

IC_{50} values are the geometric mean 50% inhibitory concentrations. IC_{50} threshold for resistance to chloroquine >100nM; threshold resistance to artesunate >20nM; r^2 indicates correlations of antiplasmodial activity of the drug assessed by microscopy and sybr green assay methods.

CHAPTER FIVE

`5.0 DISCUSSION

In this study, we validated factors which could influence the outcome of the in vitro drug test using the sybr green fluorescence method. The method was used further to determine the susceptibility of Ghanaian *P. falciparum* isolates to artesunate and chloroquine comparing it to the conventional Giemsa stain microscopic technique.

Corresponding to earlier publications (Jacob *et al*., 2007; Moneriz *et al*., 2009) there was a linear increase in Sybr green I fluorescence intensity with increase in parasite DNA (r^2=0.9447-0.9962) (fig 4-11). The assay indicated a quantification limit (QL) of 0.5% as against 0.7% parasitaemia, which allow the detection of ~10 parasites per microliter (Moneriz *et al*., 2009) in previous studies. Parasites quantification was regardless of the growth stage. In terms of validating the quality of the assay for high throughput screening, an average median z' value of ≥4.7 for all test wells was determined which is quite higher than the values of 0.73 and 0.95 in previous publication by (Jacob *et al.,* 2007) and which agrees with the acceptable standard z' value of ≥0.5 (Zhang *et al*., 1999; Weisman *et al*., 2006).

There was no statistical difference (p = 0.988) between fluorescence readings five minutes after the addition of sybr green and lysis buffer mix and readings after one hour of incubation of the samples after the addition of sybr green and lysis buffer mix. Incubating the samples for 3hrs indicated statistical difference in fluorescence (F= 1.35 and P= 0.257) with a coefficient of variation of 65.8% and 5617.8 standard error of means difference (s.e.d) when compared with five minutes incubation of the samples. There was also a significant difference (F=1.19 and p=0.286) between fluorescence readings for five minutes incubation after the addition of sybr green and lysis buffer mix and readings after overnight of incubation of the samples with sybr green and lysis buffer mix. The

difference in fluorescence observed at different incubation time periods has shown that, the time interval of incubation does have an influence on the performance of the sybr green assay. Freezing the samples before incubation with the sybr green indicated a marked difference (F= 3.42, P=0.078) in fluorescence with 13.3% (59877.7/4497123) as against 24.3% (56114/232354.3) (table 1) for samples incubated after the addition of sybr green/lysis buffer mixture. The results indicated that freezing the samples provided better lysis of the infected RBCs and parasites, thus releasing the DNA, which could then freely react with the Sybr green I as indicated in a similar study (Smilkstein *et al*., 2004). A 10-fold increase in fluorescence was observed after a freeze-thaw cycle, which is related to additional cell lysis. The erythrocyte lysis and parasite lysis are not complete at one hour (Smilkstein *et al*., 2004). The increase in fluorescence after a freeze-thaw step further indicates that the fluorescence at one hour is far from maximal as determined, under experimental conditions in this study.

A decreasing trend in Sybr green fluorescence with increasing haemoglobin at fixed salmon sperm DNA (1000ng/ml) was observed in this study. The decreasing trend in fluorescence observed may be as a result of quenching of the sybr green fluorescence by the haemoglobin (Quashie *et al*., 2006). A regression analysis showed a positive correlation (r^2=0.9492) with regression coefficient of -43.88 ± 4.146 (fig. 10). Two-fold serial dilution of extracted 3D7 DNA of initial concentration 0.2ng/µl after saponin treatment and washing in 1xPBS to remove all the haemoglobin, the experiment showed a linear increase in fluorescence with a corresponding increase in DNA concentration (fig 11). In other studies, removal of haemoglobin from infected red-blood cells culture (IRBC) increased considerably the fluorescent signal (Moneriz *et al*., 2009) which agrees with the findings in this study. In their study results shown for haemoglobin non-depleted samples and haemoglobin depleted in the absence or presence of 2% Triton X-100.

(Moneriz *et al*., 2009) correlates with the findings of results shown for haemoglobin non-depleted samples and haemoglobin depleted with 10% saponin and lysing the parasites followed by re-suspending the extracted DNA in 1x PBS. A significant difference (F=1.26, P=0.282) in the mean fluorescence of sybr green in the experiment involving extracted DNA and experiment involving varying haemoglobin was observed. Observation of the two experiments shows that the hematocrit is a variable that needs to be addressed since IC_{50} values have been found to increase dramatically as the hematocrit increases (Kamchonwongpaisan *et al*., 1994). In the work of Kamchonwongpaisan and others (Kamchonwongpaisan *et al*., 1994) they have indicated that the increase in IC_{50} values for parasites infecting variant erythrocytes was also related to the decrease in parasite accumulation due to parasite seclusion by high haematocrit, indicating that drug accumulation capacity of the parasite also has a role in determining drug sensitivity. The obtained results after validation of the assay in this study indicates that the assay is adaptable to a wide range of conditions as reported in the study by Smilkstein and colleague (Smilkstein *et al*., 2004) during development of the MSF assay. Successful assays were performed with starting parasitemia levels ranging from 0.2 to 2%, hematocrit levels ranging from 1 to 4%, asynchronous and synchronized cultures, growth durations of 24 h (schizont maturation assay) to 72 h, and plate reading at a variety of intervals after addition of the dye.

Sybr green assay and microscopy methods were employed in determining *in vitro* drug sensitivity of *P. falciparum* clinical isolates to artesunate (AS) and chloroquine (CQ) showing similar results (Fig 12 and 13). All successfully tested parasite isolates were sensitive to artesunate and chloroquine. Isolates subjected to chloroquine and artesunate two-fold serial dilution dose indicated a percentage growth of 63% and 37% respectively with IC_{50} values (table 4&5) well below the threshold for resistance. A positive correlation

(r^2 =0.1182, P=0.1378, CI;-2.563 to 17.10) suggest a strong antiplasmodial activity of chloroquine by both methods likewise for artesunate (r^2 =0.0098, P=0.6772, CI; -21.38 to 14.21,) (table 6).

5.1 Conclusions and Recommendations

The results of this study show that sybr green assay has a strong linear detection of parasite DNA at various concentrations. Factors that could have an influence on its performance have also been demonstrated. For all of the isolates tested, the sybr green assay method yielded identical results compared with the gold standard Giemsa stain microscopy method. The sybr green assay proved successful for cultured 3D7 strains at 7.8% to 0.004% levels of parasitaemia and 0.2ng/µl to 0.003ng/µl of extracted DNA as well as its applicability to clinical isolates. The fluorometer used makes it easier to read a large number of plates within a short time hence the sybr green assay is an easy to perform alternative, due to the substantial amount of time spent in reading slides making the microscopy method somehow a cumbersome procedure. Field isolates should be used to further validate the assay to address the issue of specificity since the sybr green binds to all DNA. Based on the results, it is therefore concluded that the Sybr green assay is a reliable tool without time consumption and hazards of other screening assays and can be used for malaria drug susceptibility monitoring on the field.

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